รายงานวิจัยฉบับสมบูรณ์

โครงการองค์ประกอบทางเคมีและฤทธิ์ชีวภาพจากรากและลำต้นของคันทรง

โดย ดร. รัศมี เหล็กพรม

มิถุนายน 2560

สัญญาเลขที่ TRG5880005

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และต้นสังกัดไม่จำเป็นต้องเห็นด้วยเสมอไป)

สัญญาเลขที่ TRG5880005

โครงการ โครงการองค์ประกอบทางเคมีและฤทธิ์ชีวภาพจากรากและลำต้นของคันทรง

บทคัดย่อ

การแยกองค์ประกอบทางเคมีของพืชสมุนไพรไทยจากต้นคันทรงด้วยวิธีทางโครมาโทกราฟี แยกได้สารองค์ประกอบทั้งหมด 21 สาร โดยได้จากส่วนสกัดหยาบของกิ่งคันทรง 16 สาร ประกอบไปด้วยสาร ceanothic acid (**1**), granulosic acid (**2**), zizyberenalic acid (**3**), colubrinic acid (**4**), alphitolic acid (**5**) and betulinic acid (**6**), stigmast-5-en-7-one (**7**), 6β-hydroxystigmast-4-en-3-one (**8**), stigmast-4-ene-3, 6-dione (**9**), ergosterol peroxide (**10**), β-sitosterol (**11**) and β-sitosterol-3-*O*-glycoside (**20**), 2-hydroxy-5-methoxybenzoic acid (**12**), (-)-auranamide (**13**), D-phenylalanine, *N*-benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester (**14**), isointermedeol (**15**) และสาร 3'',2'''-*O*-acetylcolubrin (**16**) สำหรับการแยกส่วนสกัดหยาบของรากคันทรงประกอบไปด้วยสารใหม่กลุ่มไทรเทอร์พีนซนิด ceanothane จำนวน 2 สาร คือ 3β -benzoylcolubenzoylatic acid (**17**) และ 3β - acetylcolubenzoylatic acid (**18**) และสารที่รายงานโครงสร้างแล้ว 9 สาร คือ สาร ceanothic acid (**1**), granulosic acid (**2**), zizyberenalic acid (**3**), alphitolic acid (**5**), betulinic acid (**6**), β-sitosterol-3-*O*-glycoside (**20**), 3'',2'''-*O*-acetylcolubrin (**16**), 3''-*O*-acetylcolubrin (**19**) และสาร squalene (**21**) สำหรับการพิสูจน์โครงสร้างของสารเหล่านี้ อาศัยเทคนิคทางสเปกโทรสโกปี (UV, IR, HR-ESI-TOF-MS, ^{1}H NMR ^{13}C NMR และ 2D NMR) จากการทดสอบฤทธิ์ทางชีวภาพของสารที่แยกได้ พบว่า สาร **3**, **10**, **17**, **18** และสาร **21** มีฤทธิ์ต้านเชื้อมาลาเรีย *Plasmodium falciparum* ที่ค่า IC_{50} ระหว่าง 2.32 ถึง 4.67 µg/mL ในขณะที่สาร **5** และสาร **18** มีฤทธิ์ต้านเชื้อวัณโรค *Mycobacterium tuberculosis* ที่ค่า MIC 50.00 and 6.25 µg/mL นอกจากนี้สาร **3**, **5**, **6**, **10**, **14**, **16-19** และ **21** มีความเป็นพิษต่อเซลล์มะเร็ง KB, NCI-H187 และ MCF-7 ที่ค่า IC_{50} ระหว่าง 8.32 ถึง 46.72 µg/mL

คำสำคัญ: ต้นคันทรง วงศ์พุทรา ฤทธิ์ต้านเชื้อมาลาเรีย เป็นพิษต่อเซลล์มะเร็ง ฤทธิ์ต้านเชื้อวัณโรค

สัญญาเลขที่ TRG5880005

โครงการ Chemical constituents and bioactivity from roots and stem of *Colubrina asiatica*

Abstract

The phytochemical investigation of Thai medicinal plant, *Colubrina asiatica*, led to the isolation of twenty-one compounds. The crude extracts from the branches of *C. asiatica* gave sixteen compounds, ceanothic acid (**1**), granulosic acid (**2**), zizyberenalic acid (**3**), colubrinic acid (**4**), alphitolic acid (**5**) and betulinic acid (**6**), stigmast-5-en-7-one (**7**), 6β-hydroxystigmast-4-en-3-one (**8**), stigmast-4-ene-3,6-dione (**9**), ergosterol peroxide (**10**), β-sitosterol (**11**) and β-sitosterol-3-*O*-glycoside (**20**), 2-hydroxy-5-methoxybenzoic acid (**12**), (-)-auranamide (**13**), D-phenylalanine, *N*-benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester (**14**), isointermedeol (**15**) and 3'',2'''-*O*-acetylcolubrin (**16**). The crude extracts from the roots of *C. asiatica* yielded two new ceanothane triterpenes, 3β - benzoylcolubenzoylatic acid (**17**) and 3β - acetylcolubenzoylatic acid (**18**), along with nine known compounds, ceanothic acid (**1**), granulosic acid (**2**), zizyberenalic acid (**3**), alphitolic acid (**5**), betulinic acid (**6**), β-sitosterol-3-*O*-glycoside (**20**), 3'',2'''-*O*-acetylcolubrin (**16**), 3''-*O*-acetylcolubrin (**19**) and squalene (**21**). Their structures were elucidated by spectroscopic methods (UV, IR, HR-ESI-TOF-MS, ^{1}H NMR ^{13}C NMR and 2D NMR). Compounds **3**, **10**, **17**, **18** and **21** showed antimalarial activity against *Plasmodium falciparum* with IC_{50} values ranging from 2.32 to 4.67 µg/mL, while compounds **5** and **18** showed antimycobacterial activity against *Mycobacterium tuberculosis* (MIC 50.00 and 6.25 µg/mL, respectively). In addition, compounds **3**, **5**, **6**, **10**, **14**, **16**-**19** and **21** showed cytotoxicity against cancer cell lines, KB, NCI-H187 and MCF-7 with IC_{50} values ranging from 8.32 to 46.72 µg/mL.

Keyword: *Colubrina asiatica*, Rhamnaceae, antimalarial, cytotoxicity, antimycobacterial

สัญญาเลขที่ TRG5880005

โครงการ องค์ประกอบทางเคมีและฤทธิ์ชีวภาพจากรากและลำต้นของคันทรง

Executive Summary

The Rhamnaceae family contains approximately 900 species and is widely spread throughout the tropical and subtropical regions of the world, consisting of small shrubs and occasionally trees. The genus *Colubrina* comprises about 30 species and is a rich source of biologically active compounds. Therefore, they play an important role in economy and two species have been found in Thailand, *C. asiatica* and *C. pubescens*. According to previously reported literature, the biological activities of compounds isolated from the genus *Colubrina* mostly originate from saponins, triterpenoids, flavonoids, alkaloids, phenolic compounds and essential oils.

Colubrina asiatica (L.) Brongn (Rhamnaceae) is a scrambling shrub that reaches up to 1-4 m in height, growing widely in Southeast Asia, tropical Australia and the Pacific Islands. Its synonym is *Ceanothus asiaticus* L. and is known as “Khan song”, or “Kan thoeng” in Thai. The leaves and bark are used traditionally as a decoction for the treatment of skin diseases, the roots as a treatment against canker sore and malnutrition, fruits as a fish poison and as a soap substitute, respectively. In southern Thailand, it is cooked atop of steamed fish. Previous investigations of the leaves of *C. asiatica* led to the isolation of Jujubogenin glycosides, colubrine and colubrinoside, several flavonoid glycosides, as well as the bisbenzylisoquinoline alkaloid *O*-methyldauricine in the bark. However, no phytochemical constituents from roots and stems of *C. asiatica* have been reported. Our continuous efforts to the phytochemistry research on the branchs and the roots of this plant led to the isolation of two new compounds (**17**-**18**) and nineteen known compounds (**3**-**16** and **19**-**21**). Their structures were elucidated by spectroscopic methods (UV, IR, HR-ESI-TOF-MS, ^{1}H NMR ^{13}C NMR and 2D NMR). Compounds **3**, **10**, **17**, **18** and **21** showed antimalarial activity against *Plasmodium falciparum* with IC_{50} values ranging from 2.32 to 4.67 µg/mL, while compounds **5** and **18** showed antimycobacterial activity against *Mycobacterium tuberculosis* (MIC 50.00 and 6.25 µg/mL, respectively). In addition, compounds **3**, **5**, **6**, **10**, **14**, **16**-**19** and **21** showed cytotoxicity against cancer cell lines, KB, NCI-H187 and MCF-7 with IC_{50} values ranging from 8.32 to 46.72 µg/mL.

Output: The research outcome includes three international publication papers.

1. Sangsopha, W., Kanokmedhakul, S., Lekphrom R., Kanokmedhakul, K. Chemical constituents and biological activities from branches of *Colubrina asiatica*. Natural Product Research DOI: 10.1080/14786419.2017.1320787. (IF = 1.057)
2. Lekphrom, R., Kanokmedhakul, K., Sangsopha, W., Kanokmedhakul, S. A new coumarin from the roots of *Micromelum minutum*. Natural Product Research 2016, 30, 2383-2388. (IF = 1.057)
3. Sangsopha, W., Lekphrom, R., Kanokmedhakul, S., Kanokmedhakul, K. Cytotoxic and antimalarial constituents from aerial parts of *Sphaeranthus indicus*. Phytochemistry Letter 2016, 17, 278-281. (IF = 1.353)

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Introduction

Colubrina asiatica (L.) Brongn (Rhamnaceae) is a scrambling shrub that reaches up to 1-4 m in height, growing widely in Southeast Asia, tropical Australia and the Pacific Islands. Its synonym is *Ceanothus asiaticus* L. and is known as “Khan song”, or “Kan thoeng” in Thai. The leaves and bark are used traditionally as a decoction for the treatment of skin diseases, the roots as a treatment against canker sore and malnutrition, fruits as a fish poison and as a soap substitute, respectively. In southern Thailand, it is cooked atop of steamed fish.[1,2]

Figure 1 *Colubrina asiatica* (L.) Brongn

Previous reports of compounds from the leaves of *C. asiatica* have been reported in three publications, focusing only on its leaves and bark. The isolated structures are shown in Figure 2.

In 1970, Tschesche and coworkers[3] investigated the alkaloid extracts of the bark of *C. asiatica* and reported its major alkaloid, *O*-methyl-dauricine (**I**). In 1983, Wagner and coworkers[4] investigated the bark of *C. asiatica* and reported two saponins, colubrinoside (**II**) and colubrine (**III**). Both saponins inhibit the spontaneous motility of mice, even at low doses (1 mg/kg), they show an antagonistic effect on amphetamine and exert a synergistic activity on chlordiazepoxide. In 2000, Lee and coworkers[5] reported three new 3''-*O*-acetylcolubrin (**IV**), 3'',2'''-*O*-diacetylcolubrin (**V**) and 3''-O-acetyl-6''-*O*-*trans*-crotonylcolubrin (**VI)** along with three known compounds, colubrine (**III**), Kaempferol 3-*O*-rutinoside (**VII**) and rutin (**VIII**).

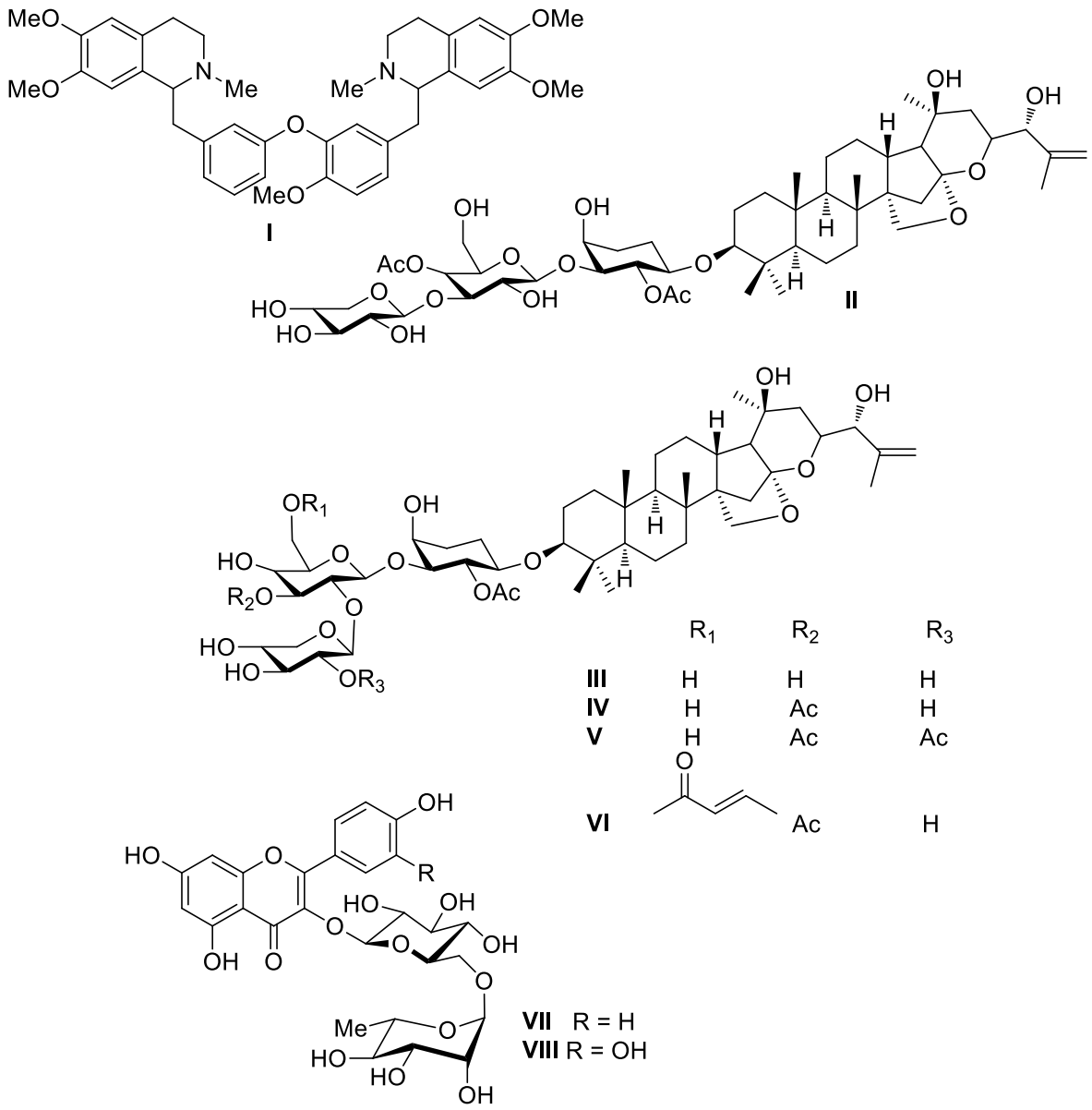

Figure 2 Chemical constituents from *Colubrina aciatica*

As part of our research on Thai medicinal plants containing potential bioactive compounds, air-dried branches and roots of *C. asiatica* were investigated. Both EtOAc and MeOH extracts showed activity against *Plasmodium falciparum*, *Microbacterium tuberculosis* and cytotoxicity towards three cancer cell lines. We report herein the first isolation, characterization and biological activities of twenty-one isolated compounds from the branches and the roots of this plant.

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Experimental

Plant material

The branches of *C. asiatica* were collected at Amphur Muang, Mahasarakham Province, Thailand, in June 2014. The plant material was identified by Prof. Pranom Chantaranothai, Department of Biology, Khon Kaen University, Thailand, where a voucher specimen (R. LekphromKKU005) was deposited.

General experimental procedures

Melting points were determined using Electrothermal IA9200 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK). Optical rotations were measured on a JASCODIP-1000 digital polarimeter (JASCO Inc., USA) and UV spectra were recorded using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). IR spectra were obtained using a Bruker Tenser 27 spectrophotometer (Bruker, Germany). NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer (Varian Inc., USA) using $CDCl_3$, CD_3OD and DMSO-d_6 as solvents. The internal standards were referenced from the residue of those solvents. The HR-ESI-TOF-MS were recorded on a Bruker micrOTOF mass spectrometer (Brucker, Germany). Column chromatography was carried out on MERCK silica gel 60 (230–400 mesh) (Merck, Darmstadt, Germany). Thin-layer chromatography was carried out with pre-coated MERCK silica gel 60 PF254 (Merck, Darmstadt, Germany); the spots were visualised under UV light (254 and 365 nm) and further by spraying with anisaldehyde and then heating until charred.

Extraction and Isolation of branches of C. asiatica

Air-dried branches of *C. asiatica* (1.8 kg) were ground to powder and then were extracted at room temperature with EtOAc and MeOH three times each (3L x 3). Removal of solvents under reduced pressure gave crude EtOAc (23.1 g) and MeOH (52.8 g) extracts. The extraction scheme is shown is Figure 3.

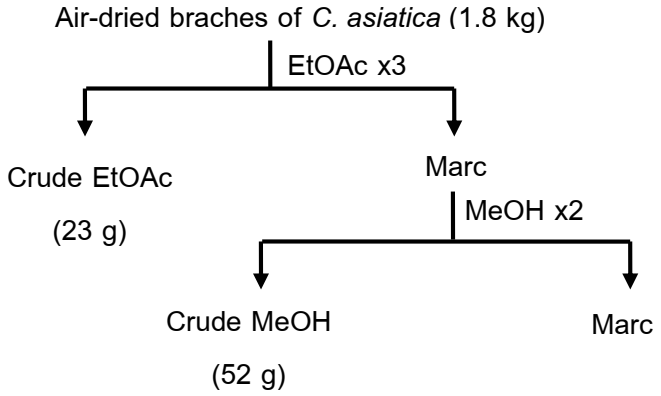


Figure 3 Solvent extraction scheme of branches of *C. asiatica*

The crude EtOAc extract (23.1 g) was separated over silica gel column chromatography (CC), eluted with a gradient systems of EtOAc-hexane (0:100-100:0) and MeOH-EtOAc (0:100-100:0) to give seven fractions, bCE_1-bCE_7. Fraction bCE_2 was subjected to silica gel flash column chromatography (FCC), eluted with a gradient system of EtOAc-hexane (1:10), to give ten subfractions, $bCE_{2.1}$-$bCE_{2.10}$. Subfraction $bCE_{2.7}$ was further purified by preparative TLC using CH_2Cl_2-hexane (17:3) as developing solvents to yield **15** as a colorless oil (10.5 mg) and **7** as an amorphous solid (142.8 mg). Solid in subfraction $bCE_{2.9}$ was recrystallized with hexane to yield **9** as a white solid (5.5 mg). Subfraction $bCE_{2.10}$ was purified to give **3** as a white solid (5.2 mg). Fraction bCE_3 was separated by silica gel FCC, eluted with a gradient system of MeOH-CH_2Cl_2 (0:100) to give ten subfractions, $bCE_{3.1}$-$bCE_{3.10}$. Subfraction $bCE_{3.6}$ was submitted to silica gel FCC, eluting with a gradient system of MeOH-CH_2Cl_2 (1:100) to give nine subfractions, $bCE_{3.6.1}$-$bCE_{3.6.9}$. Subfraction $bCE_{3.6.1}$, $bCE_{3.6.8}$ and $bCE_{3.6.9}$ was purified to obtain **6** (37.2 mg), **4** (5.0 mg) and **1** (206.6 mg) as white solids. Subfraction $bCE_{3.6.4}$ was submitted to silica gel FCC, using EtOAc-hexane (3:7) to give **8** (15.5 mg) and **10** (11.7 mg) as white solids. Subfraction bCE3.6.2 was submitted to silica gel FCC, using EtOAc-n-hexane (3:7) to give 8 (15.5 mg) and 10 (11.7 mg) as white solids. Subfraction bCE_5 was separated by silica gel FCC, eluting with a gradient system of EtOAc-CH_2Cl_2 (0:100), to give nine subfractions, $bCE_{5.1}$-$bCE_{5.9}$. Purification of subfractions $bCE_{5.1}$, $bCE_{5.3}$, $bCE_{5.5}$ and $bCE_{5.9}$ gave **11** (23.1 mg), **13** (18.2 mg), **14** (12.4 mg) and **5** (30.5 mg) as white solids. Subfraction bCE_6 was submitted to silica gel FCC, eluting with EtOAc-CH_2Cl_2 (1:5), to give ten subfractions, $bCE_{6.1}$-$bCE_{6.10}$.

Purification of subfractions $bCE_{6.4}$ and $bCE_{6.10}$ yielded **12** (5.9 mg) and **2** (9.5 mg) as white solids, respectively.

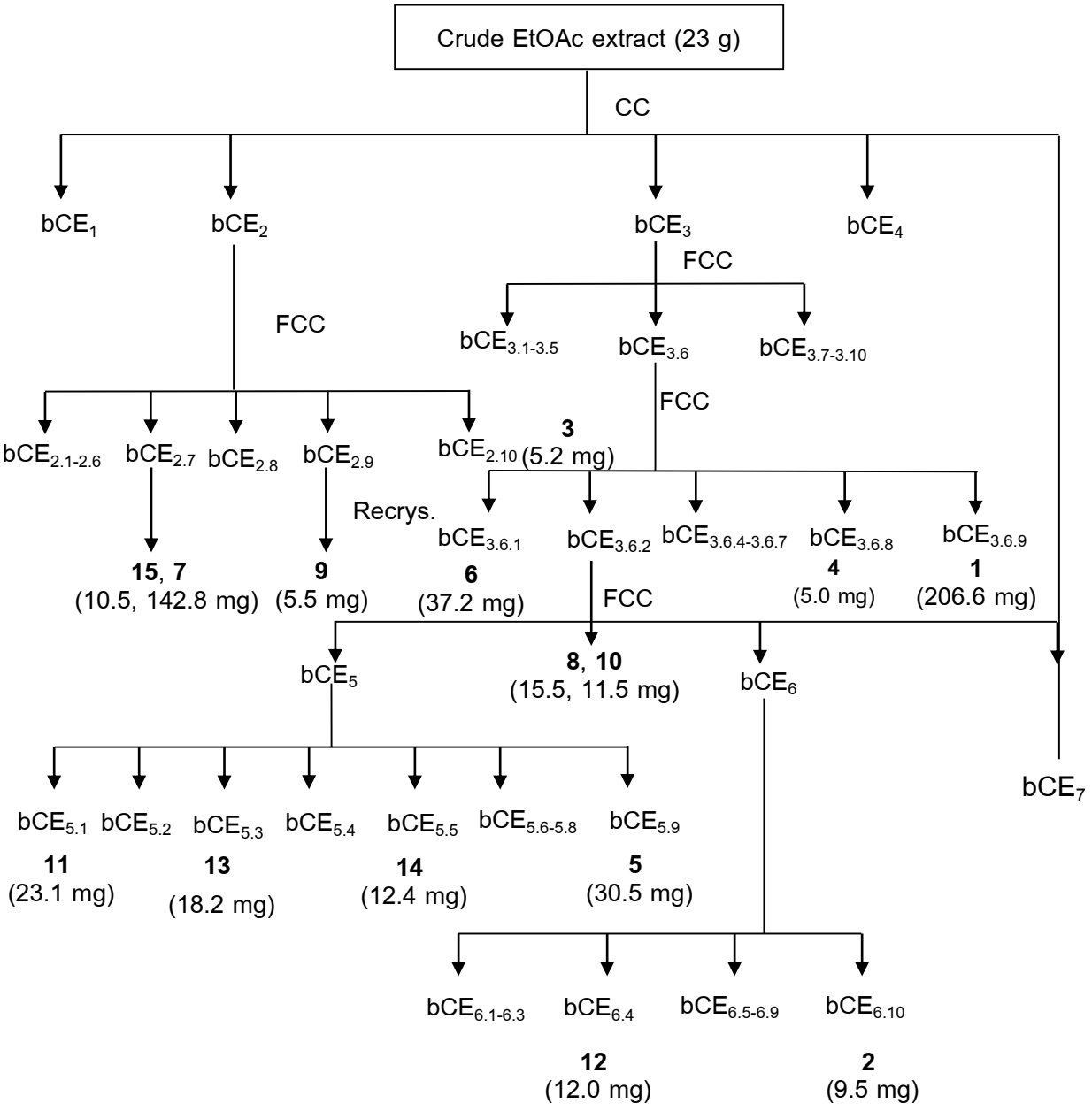


Figure 4 Separation scheme of crude EtOAc extract of branches of *C. asiatica*

The crude MeOH extract (52.8 g) was separated using silica gel CC with MeOH-acetone-CH_2Cl_2 (2:1:1) to give eight fractions, bCM_1-bCM_8. Subfraction bCM_1 was further separated by silica

gel FCC and eluted with a gradient system of MeOH-EtOAc (0:100) to give **16** (54.5 mg) as an amorphous solid.

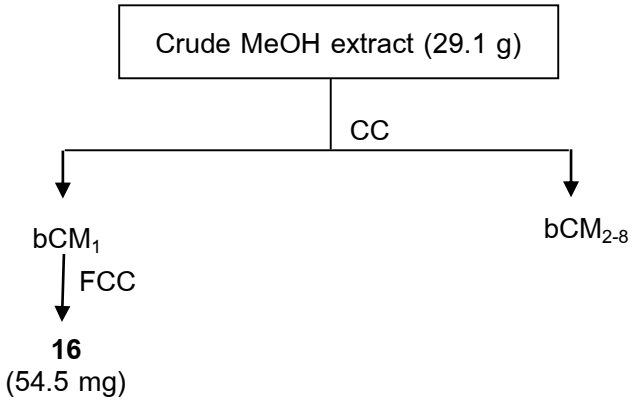


Figure 5 Separation scheme of crude MeOH extract of branches of *C. asiatica*

Extraction and Isolation of roots of C. asiatica

Air-dried roots of *C. asiatica* (2.0 kg) were ground to powder and then extracted at room temperature with EtOAc and MeOH three times each (3L x 3). Removal of solvents under reduced pressure gave crude EtOAc (35.0 g) and MeOH (155.0 g) extracts. The extraction scheme is shown is Figure 6.

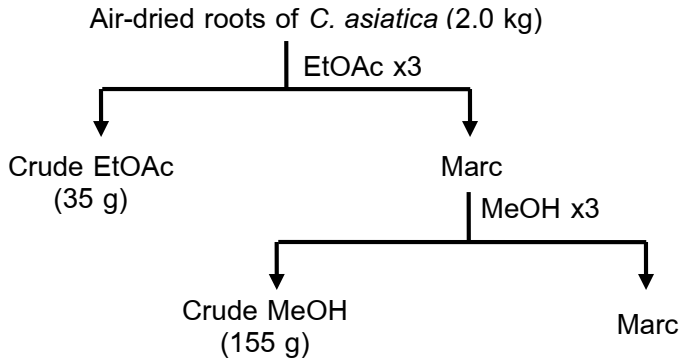


Figure 6 Solvent extraction scheme of roots of *C. asiatica*

The crude EtOAc extract (35.0 g) was separated over silica gel column chromatography (CC), eluted with a gradient systems of EtOAc-*n*-hexane (0:100-100:0) and MeOH-EtOAc (0:100-100:0) to give seven fractions, rCE_1-rCE_7. Fraction rCE_1 was subjected to silica gel flash column chromatography (FCC), eluted with a gradient system of EtOAc-*n*-hexane (1:19) to give eight subfractions, $rCE_{1.1}$-$rCE_{1.8}$. Subfraction $rCE_{1.1}$ and $rCE_{1.5}$ led to **21** (15.5 mg) and **3** (59.8 mg) as a yellow oil and white solids, respectively. Subfraction $rCE_{1.8}$ was separated by silica gel FCC, eluting with a gradient system of MeOH-CH_2Cl_2-*n*-hexane (2:65:33) to yield **18** as a white solid (8.0 mg). Fraction rCE_2 was subjected to silica gel FCC, eluted with a gradient system of EtOAc-*n*-hexane (1:19) to give eight subfractions, $rCE_{2.1}$-$rCE_{2.8}$. Subfraction $rCE_{2.4}$ was applied to gel FCC, eluted with a gradient system of MeOH-CH_2Cl_2-*n*-hexane (1:30:19) to give five subfractions, $rCE_{2.4.1}$-$rCE_{2.4.5}$. Subfraction $rCE_{2.4.2}$ and $rCE_{2.4.4}$ led to **17** (27.0 mg) and **5** (43.4 mg) as white solids. Fraction rCE_3 was dissolved with CH_2Cl_2 and the precipitate was filtered to obtain a white solid of **1** (1.1 g). The filtrate was evaporated over silica gel flash CC, eluted with a gradient system of EtOAc-hexane (3:7) to give **6** as a white solid (70.0 mg). Fraction rCE_5 was separated by silica gel FCC, eluted with a gradient system of EtOAc-CH_2Cl_2 (3:7) to give eight subfractions, $rCE_{5.1}$-$rCE_{5.8}$. Subfraction $rCE_{5.6}$ led to **2** (186.3 mg) as a white solid.

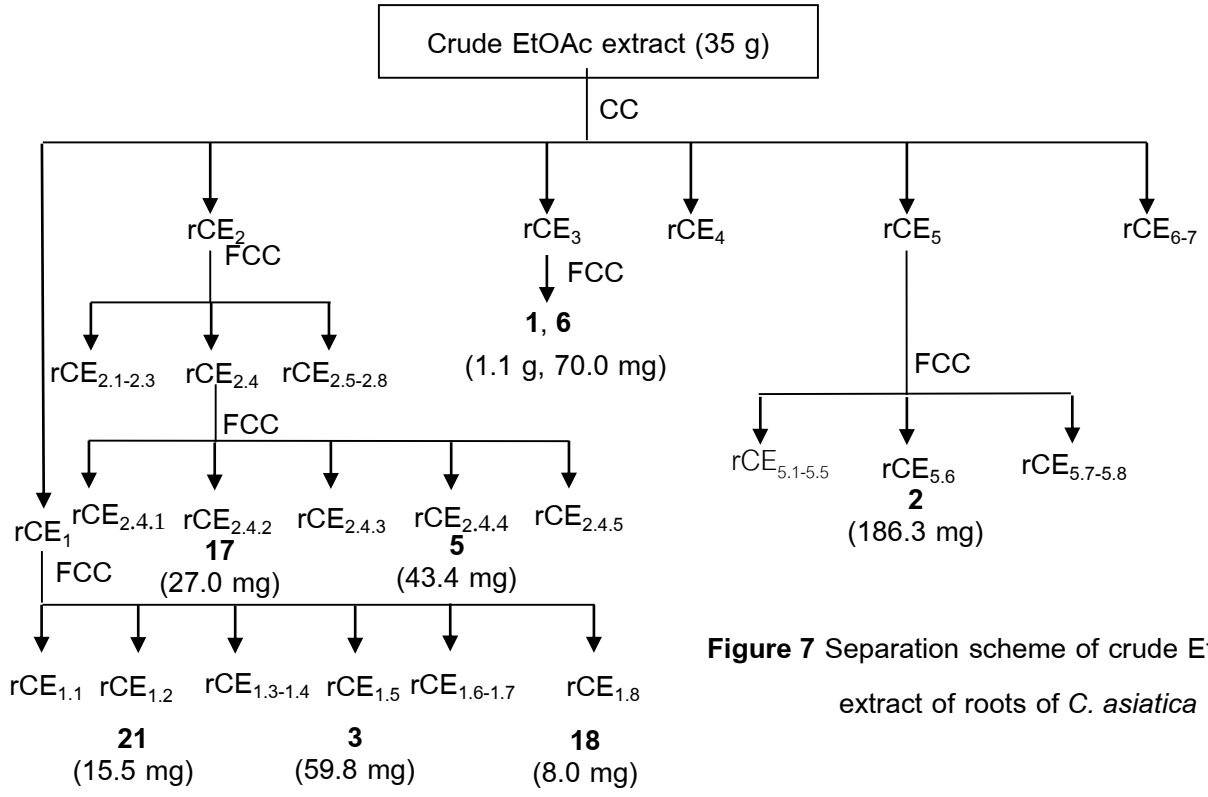


Figure 7 Separation scheme of crude EtOAc extract of roots of *C. asiatica*

The crude MeOH extract (150.0 g) was separated using silica gel CC with MeOH-CH_2Cl_2 (1:19) to give eight fractions, rCM_1-rCM_7. Subfraction rCM_2 was separated by silica gel FCC, eluted with MeOH-EtOAc-CH_2Cl_2 (1:25:24) to give seven subfrations, $rCM_{2.1}$-$rCM_{2.7}$. $rCM_{2.6}$ was separated by FCC to give four subfractions, $rCM_{2.6.3}$-$rCM_{2.6.4}$. Subfraction $rCM_{2.6.2}$ led to **20** as a white solid (10.0 mg). Subfraction $rCM_{2.6.3}$ was separated by silica gel FCC, eluted with MeOH-EtOAc-CH_2Cl_2 (2:25:23) to give **16** (60.5 mg) and **19** (20.0 mg) as amorphous solids.

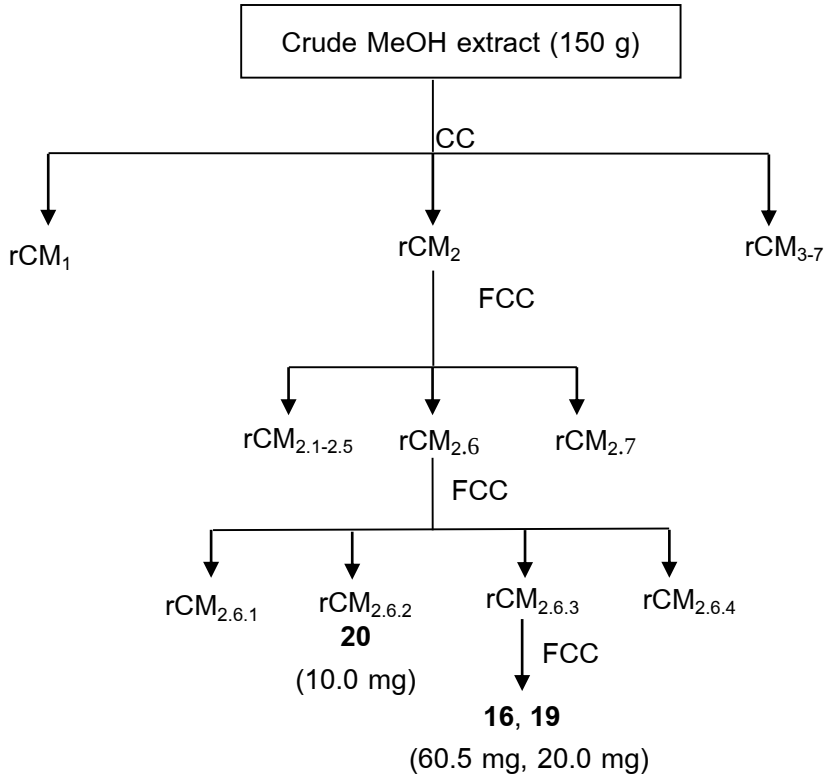


Figure 8 Separation scheme of crude MeOH extract of roots of *C. asiatica*

Results and Discussion

The phytochemical investigation of Thai medicinal plant, *Colubrina asiatica*, led to the isolation of twenty-one compounds. The crude extracts from the branches of *C. asiatica* gave sixteen compounds, including six triterpene acids: ceanothic acid (**1**),[6] granulosic acid (**2**),[6] zizyberenalic acid (**3**),[7] colubrinic acid (**4**),[8] alphitolic acid (**5**)[8] and betulinic acid (**6**)[7]; six steroids: stigmast-5-en-7-one (**7**),[9] 6β-hydroxystigmast-4-en-3-one (**8**),[10] stigmast-4-ene-3,6-dione (**9**),[11] ergosterol peroxide (**10**),[12] β-sitosterol (**11**)[9] and β-sitosterol-3-*O*-glycoside (**20**)[13]; 2-hydroxy-5-methoxybenzoic acid (**12**)[14]; two phenylanalines: (-)-auranamide (**13**) and D-phenylalanine, *N*-benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester (**14**)[15]; sesquiterpenoid: isointermedeol (**15**)[16]; two jujubogenin glycosides: 3'',2'''-*O*-acetylcolubrin (**16**).[17] The crude extracts from the roots of *C. asiatica* yielded two new ceanothane triterpenes, 3β-benzoylcolubenzoylatic acid (**17**) and 3β- acetylcolubenzoylatic acid (**18**), along with nine known compounds, including five triterpene acids: ceanothic acid (**1**), granulosic acid (**2**)[6], zizyberenalic acid (**3**),[7] alphitolic acid (**5**)[8] and betulinic acid (**6**)[7] ; steroid: β-sitosterol-3-*O*-glycoside (**20**)[13]; two jujubogenin glycosides: 3'',2'''-*O*-acetylcolubrin (**16**) and 3''-*O*-acetylcolubrin (**19**)[17]; triterpenoid: squalene (**21**).[18] (Figure 12)

Compound **17** was obtained as white solids and its molecular formula $C_{45}H_{56}O_8$ was deduced from HRESITOFMS (m/z 747.3865 $[M+Na]^+$). The IR spectrum showed the presence of ester (1720 cm^{-1}) and aromatic (1453 cm^{-1}) groups. The ^{1}H NMR spectrum (Table 1) showed the presence of 2-methyl-3β,7-dibenzoyl substituted ceanothic acid (**1**) skeleton as a singlet at 3.75 (3H, s) and aromatic protons at δ 8.01-7.33 (10H, ArH); terminal methylene protons at δ 4.75 and 4.64 (each 1H, brs); three methine protons handing esters at δ 5.37 (1H, s, H-3), 5,30 (1H, dd, *J* = 10.4, 4.8 Hz, H-7) and 2.77 (1H, s, H-1); and six quaternary methyl groups δ 1.69, 1.32, 1.31, 1.19 1.09 and 0.96. The ^{13}C NMR and HSQC experiment showed a pattern of triterpene skeleton with a series of six methyl, eight methylene, eight methane and eight quaternary carbons. The ^{13}C NMR also showed the presence of four carbonyl carbons δ 181.5, 173.6, 165.8, 165.7; twelve aromatic carbons δ 133.1, 132.9, 131.0, 130.4, 129.5 x 4 and 128.4 x 4; and one methyl carbon δ 51.8 (Table 1). The COSY spectrum of **17** showed the connection of H-1↔H-3, H-13↔H-18, H-29↔H-30, H-2′(6′)↔H-3′′(5′)↔H-4′ and H-2′′(6′′)↔H-3′′(5′′)↔H-4′′ (Fig. 9). The HMBC spectrum of **17** showed that H-3 correlated with carbonyl of C-7′′; H-5 with C-4, C-6, C-7 and C-10; H-7 with C-8, C-14, C-26 and carbonyl of C-7′; H-26 with C-7, C-8, C-9 and C-14 (Fig. 9). NOESY spectrum (Fig. 10) showed the correction of H-2′(6′)↔H-3′′(5′)↔H-4′, H-2′′(6′′)↔H-3′′(5′′)↔H-4′, H-1↔H-25, H-29↔H-30

and particular H-7↔H-27 that confirmed a benzoyl group connected to β-C-7 of the proposed structure. Therefore, compound **17** was assigned as a new ceanothane-type triterpenoid, **3β-Benzoylcolubenzoylatic acid**.

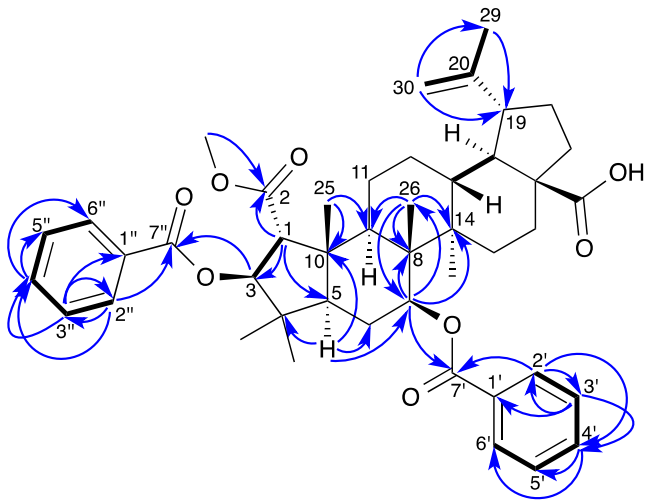


Figure 9 COSY (bold line) and selected HMBC (H→C) correlations of **17**

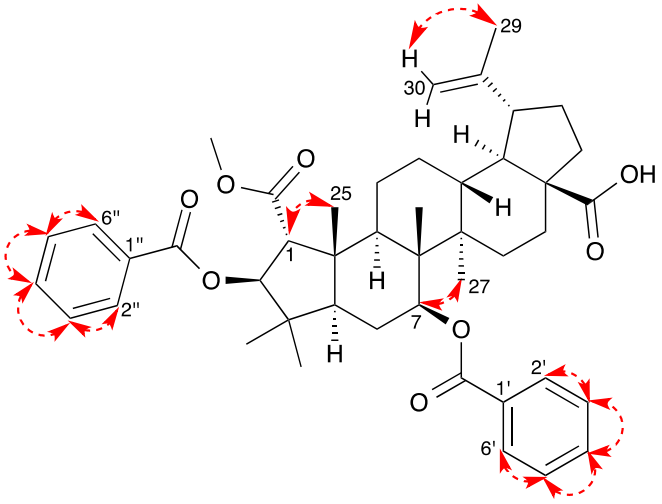


Figure 10 Key NOESY (·····) correlations of **17**

Compound **18** was obtained was white solids and its molecular formula $C_{40}H_{54}O_8$ was deduced from HRESITOFMS (m/z 685.3710 $[M+Na]^+$). The IR spectrum showed the presence of ester (1722 cm^{-1}) and aromatic (1454 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra (Table 1) were greatly similar to **18**, except for the 3β-substituted acetyl group [δ2.04 (3H, s/3-CO<u>C</u>H_3 20.9) and δ of carbonyl 3-<u>C</u>OCH_3 170.3]. NOESY of H-7/H-27 correction would be related to **18**. Thus, compound **18** was assigned as a new **3β-Acetylcolubenzoylatic acid**.

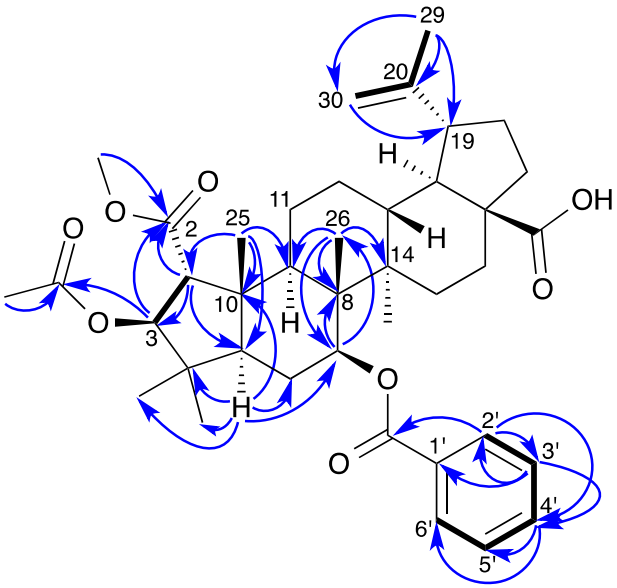


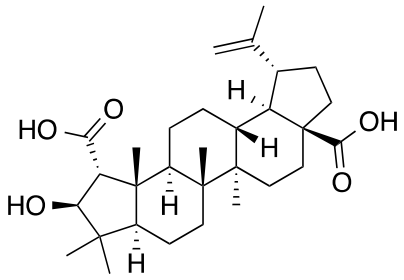
Figure 11 COSY (bold line) and selected HMBC (H→C) correlations of **18**

Table 1 ^{1}H and ^{13}C NMR spectral data for compounds **17** and **18** ($CDCl_3$)

No.	**17**		**18**	
	δ_H	δ_C	δ_H	δ_C
1	2.77 brs	62.8	2.61 brs	62.6
2		173.6		173.6
3	5.37 brs	85.7	5.12 brs	85.2
4		43.1		42.9
5	2.04 m	52.8	1.92 m	52.7
6	1.92 m, 1.73 m	25.3	1.84 m, 1.74 m	25.3
7	5.30 dd (10.4, 4.8)	77.3	5.26 dd (10.0, 3.2)	77.2
8		46.2		46.2
9	1.69 m	44.4	1.65 m	44.3
10		49.2		49.1
11	1.63 m	23.3	1.58 m	23.2
12	1.63 m	25.3	1.62 m	25.3
13	2.45 m	39.0	2.23 dt (, 3.2)	38.9
14		44.2		44.2
15	1.07 m	31.9	1.04 m	31.6
16	2.11 m, 1.43 m	32.5	2.08 m, 1.39 m	32.4
17		55.9		55.8
18	1.60 m	48.9	1.56 m	48.9
19	3.00 dt (10.0, 3.6)	47.1	2.97 m (10.8, 4.4)	47.0
20		150.0		150.0
21	1.44 m	30.6	1.39 m	30.5
22	1.97 m	37.1	1.95 m	37.1
23	1.31 s	30.3	1.23 s	30.2
24	0.96 s	19.8	0.83 s	19.4[c]
25	1.19 s	17.8	1.07 s	17.6
26	1.32 s	12.2	1.28 s	12.2
27	1.09 s	15.1	1.06 s	15.0
28		181.5		180.1
29	1.69 s	19.5	1.68 s	19.5[c]
30	4.75 brs, 4.64 brs	109.8	4.73 brs, 4.63 brs	109.8
1'		130.4[a]		131.0
2'-6'	8.01-7.33 (5H, ArH)	132.9[b], 129.5, 128.4	8.00-7.42 (5H, ArH)	132.8, 129.5, 128.4
C=O (1')		165.7		165.6
1"		131.0[a]	-	-
2"-6"	8.01-7.33 (5H, ArH)	133.1[b], 129.5, 128,4	-	-
3-$COCH_3$	-	-	2.04 s	170.3, 20.9
C=O (1")		165.8	-	-
2-OCH_3	3.75 s	51.8	3.71 s	51.7

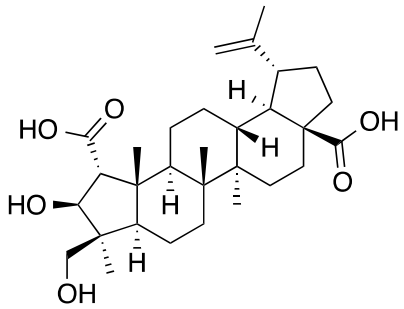
[a, b, c] may be interchangeable.

Compound 1 was obtained white solid, mp 354-356 ^{o}C. The IR spectrum showed a characteristic absorption bands of hydroxyl group at 3330 cm^{-1}. A set of absorption band at 2935, 2869 cm^{-1} were characterized to aliphatic C-H stretching, while their corresponding bending vibration appeared at 1454 and 1316 cm^{-1}. A strong absorption band at 1685 cm^{-1} was assigned to C=O stretching of a caboxyl group. A medium absorption band at 1221 cm^{-1} was assigned to C-O stretching. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **1** (Table 2 and 3) and comparison with the corresponding values reported in the literature,[6] the structure was assigned as **Ceanothic acid**.



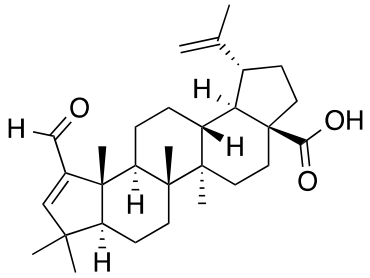
Ceanothic acid (1)

Compound 2 was obtained as white solid. Its IR spectrum showed the presence of hydroxyl groups at 3410 cm^{-1} and and carboxyl group at 1714 cm^{-1}. Based on the ^{1}H and ^{13}C NMR data of compound **2** (Table 2 and 3) supported by analysis of the two-dimensional NMR spectra and a comparison with the corresponding values reported in the literature,[6] the structure was assigned as **Granulosic acid**.



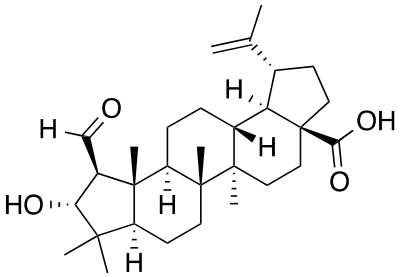
Granulosic acid (2)

Compound 3 was obtained as white solid. Its IR spectrum showed the presence of hydroxyl groups at 3362 cm^{-1} and and α, β-unsaturated aldehyde at 1695 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **3** (Table 2 and 3) and comparison with the corresponding values reported in the literature,[7] the structure was assigned as **Zizyberenalic acid**.



Zizyberenalic acid (3)

Compound 4 was obtained as white solid. Its IR spectrum showed the presence of hydroxyl groups at 3362 cm^{-1} and and α, β-unsaturated aldehyde at 1695 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **4** (Table 2 and 3) and comparison with the corresponding values reported in the literature,[8] the structure was assigned as **Colubrinic acid**.



Colubrinic acid (4)

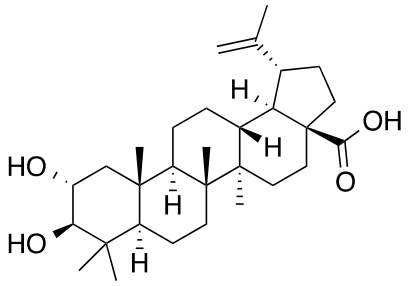
Table 2 ^{1}H NMR data of compound **1**, **2**, **3** and **4** (400 MHz)

No.	**1** ($CDCl_3$+CD_3OD)	**2** ($CDCl_3$+CD_3OD)	**3** ($CDCl_3$)	**4** ($CDCl_3$+CD_3OD)
1	2.46 (s)	2.53 (s)		
2			9.67 (s)	9.85 (d, 4.4)
3	4.08 (s)	4.19 (s)	6.54 (s)	4.22 (d, 8.4)
5	1.61 (m)	1.70 (m)	1.46 (m)	
6	1.28 (m)	1.28 (m)	1.46 (m)	
7	1.28 (m)	1.26 (m)	1.41 (m)	
9	1.67 (m)	1.61 (m)	1.88 (dd, 12.8, 2.4)	
11	1.45 (m)	1.44 (m)	2.04 (m)	
12	1.61 (m)	1.62 (m)	1.62 (m)	
13	2.17 (m)	2.15 (m)	2.14 (m)	
15	1.48 (m), 1.09 (m)	1.41 (m), 1.08 (m)	1.24 (m)	
16	2.17 (m)	2.17 (m)	2.30 (m), 2.41 (m)	
18	1.50 (m)	1.48 (m)	1.58 (m)	
19	2.93 (m)	2.92 (m)	3.00 (m)	2.94 (m)
21	1.89 (m)	1.88 (m)	2.03 (m), 1.46 (m)	
22	1.68 (m)	1.88 (m)	2.00 (m)	
23	0.86 (s)	1.22 (s)	0.95 (s)	0.95 (s)
24	1.04 (s)	4.13 (d, 11.2), 3.15 (d, 10.8)	1.14 (s)	0.95 (s)
25	1.01 s	1.09 (s)	1.12 (s)	0.90 (s)
26	0.90 s	0.87 (s)	0.97 (s)	0.86 (s)
27	0.91 s	0.88 (s)	0.96 (s)	0.85 (s)
29	4.65 (brs), 4.51 (brs)	4.64 (brs), 4.50 (brs)	4.72 (brs), 4.59 (brs)	4.69 (brs), 4.55 (brs)
30	1.61 s	1.62 (s)	1.66 (s)	1.63 (s)

Table 3 ^{13}C NMR data of compound **1**, **2**, **3** and **4** (100 MHz)

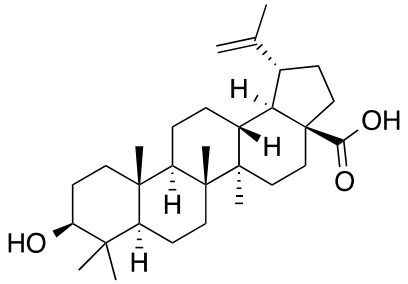
No.	**1** ($CDCl_3$+CD_3OD)	**2** ($CDCl_3$+CD_3OD)	**3** ($CDCl_3$)	**4** ($CDCl_3$+CD_3OD)
1	65.6	65.1	157.3	73.0
2	177.5	177.0	191.4	206.1
3	84.6	85.1	163.2	80.8
4	43.1	47.5	43.8	40.4
5	56.6	56.1	63.1	62.3
6	18.4	17.7	16.8	18.1
7	33.9	34.3	35.1	34.1
8	41.6	41.4	42.6	41.9
9	44.3	44.6	47.5	47.6
10	49.2	49.6	52.2	49.9
11	23.5	23.6	24.1	22.6
12	25.4	25.3	25.2	25.1
13	38.6	38.5	38.3	38.1
14	42.9	42.9	43.0	42.6
15	29.8	29.8	29.8	29.7
16	32.3	32.2	32.4	32.2
17	56.2	56.1	56.2	56.0
18	49.2	49.1	49.6	49.1
19	46.9	46.9	47.0	47.0
20	150.5	150.5	150.0	150.3
21	30.6	30.5	30.6	30.5
22	37.1	37.1	37.1	37.1
23	18.9	24.5	20.4	24.8
24	30.6	66.4	28.1	25.2
25	18.6	18.5	19.0	14.6
26	16.3	16.3	16.8	16.5
27	14.5	14.5	14.7	14.6
28	179.0	179.1	182.2	178.7
29	109.2	109.3	109.9	109.7
30	19.1	19.1	19.3	19.2

Compound 5 was obtained as white solid. Its IR spectrum showed the presence of hydroxyl groups at 3362 cm^{-1} and and carboxyl group at at 1697 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **5** (Table 4) and comparison with the corresponding values reported in the literature,[8] the structure was assigned as **Alphitolic acid**.



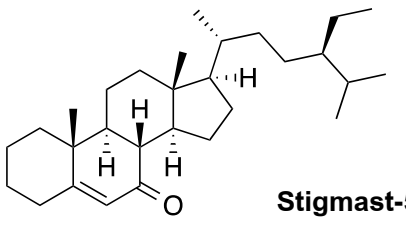
Alphitolic acid (5)

Compound 6 was obtained as white solid. Its IR spectrum showed the presence of hydroxyl groups at 3362 cm^{-1} and carboxyl group at at 1697 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **6** (Table 4) and comparison with the corresponding values reported in the literature,[7] the structure was assigned as **Betulinic acid**.



Betulinic acid (6)

Compound 7 was obtained as amorphous solid. Its IR spectrum showed the presence α, β-unsaturated ketone at 1715 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **7** (Table 5 and 6) and comparison with the corresponding values reported in the literature,[9] the structure was assigned as **Stigmast-5-en-7-one**.

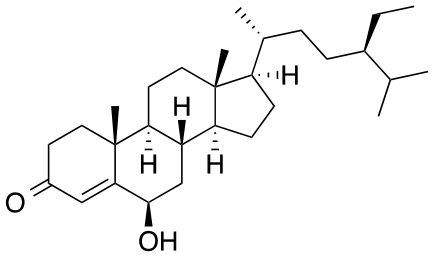


Stigmast-5-en-7-one (7)

Table 4 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of compound **5** and **6** ($CDCl_3$+CD_3OD)

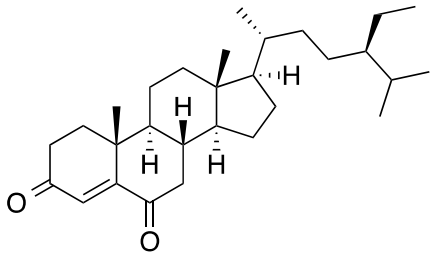
No.	**5**		**6**	
	δ_H	δ_C	δ_H	δ_C
1	1.94 (dd, 12.4, 4.0)	46.5		38.2
2	3.95 (m)	68.8		26.9
3	2.86 (d, 9.2)	83.4	3.14 (dd, 11.4, 5.6)	78.8
4		39.2		38.7
5	0.73 (s)	55.4		55.3
6	1.44 (m), 1.33 (m)	18.2		18.2
7	1.33 (m)	34.1		34.3
8		40.7		40.6
9	1.27 (m)	50.4		50.5
10		38.4		37.1
11	1.36 (m)	20.9		20.8
12	1.65 (m), 1.62 (m)	25.4		25.5
13	2.15 (m)	38.2		38.7
14		42.4		42.4
15	1.21 (m), 1.09 (m)	29.5		29.6
16	2.17 (m)	32.2		32.2
17		56.4		56.1
18	1.50 (m)	49.1		49.1
19	2.93 (dt, 10.4, 4.4)	46.9	2.95 (m)	46.9
20		150.5		150.7
21	1.36 (m)	30.5		30.5
22	1.87 (m)	37.0		37.0
23	0.93 (s)	28.3	0.90 (s)	27.8
24	0.71 (s)	16.4	0.71 (s)	15.2
25	0.81 (s)	17.2	0.78 (s)	16.0
26	0.86 (s)	15.9	0.93 (s)	15.8
27	0.90 (s)	14.5	0.92 (s)	14.5
28		179.2		179.0
29	4.65 (brs), 4.53 (brs)	109.5	4.69 (brs), 4.56 (brs)	109.4
30	1.62 (s)	19.2	1.61 (s)	19.2

Compound 8 was obtained as white solid. Its IR spectrum showed the presence α, β-unsaturated ketone at 1715 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **8** (Table 5 and 6) and comparison with the corresponding values reported in the literature,[10] the structure was assigned as **6*β*-Hydroxystigmast-4-en-3-one.**



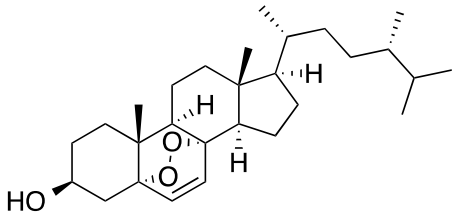
6*β*-Hydroxystigmast-4-en-3-one (8)

Compound 9 was obtained as white solid. Its IR spectrum showed the presence α, β-unsaturated ketone at 1715 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **9** (Table 5 and 6) and comparison with the corresponding values reported in the literature,[11] the structure was assigned as **Stigmast-4-ene-3,6-dione.**



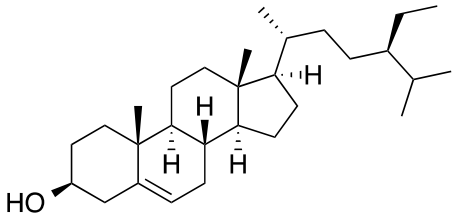
Stigmast-4-ene-3,6-dione (9)

Compound 10 was obtained white solid, mp 182-184 ^{o}C. The IR spectrum showed a broad absorption band of hydroxyl group at 3296 cm-1. The intense bands at 2953 and 2870 cm-1 were characterized as aliphatic C-H stretching, while its corresponded bending vibrations appeared at 1456 cm^{-1}. A absorption band at 1044 cm^{-1} was assigned to C-O stretching. The ^{1}H (Table 5) and ^{13}C NMR (Table 6) spectra showed the characteristic signal of a steroid unit. Comparison of the NMR spectral data, mixed-mp and mixed-TLC with the authentic sample indicated that compound **10** were **Ergosta-6,22-diene-3-ol,5,8-epidioxy or Ergosterol peroxide**.[12]



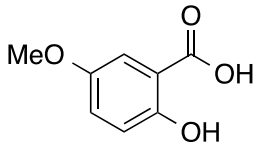
Ergosterol peroxide (10)

Compound 11 was obtained as colorless needles (mp. 136-138 $^{\circ}$C). The IR spectrum showed abroad absorption band of hydroxyl group at 3428 cm^{-1}. The intense bands at 2936, 2867 and 2849 cm^{-1} were characterized as aliphatic C-H stretching, while its corresponded bending vibrations appeared at 1464 and 1381 cm^{-1}. A medium absorption band at 1050 cm^{-1} was assigned to C-O stretching. The ^{1}H (Table 5) and ^{13}C NMR (Table 6) spectra showed the characteristic signal of a steroid unit. Comparison of the NMR spectral data, mixed-mp and mixed-TLC with the authentic phytosterol, compound 11 was concluded assigned as a ***β*-Sitostol**.[9]



***β*-Sitostol (11)**

Compound 12 was obtained as white solid (mp. 145-146 $^{\circ}$C). Its IR spectrum showed the presence of hydroxyl groups at 3282 cm^{-1} and carboxyl group at 1677 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **11** (Table 7) and comparison with the corresponding values reported in the literature, the structure was assigned as **2-Hydroxy-5-methoxybenzoic acid.**[14]

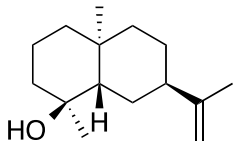


2-Hydroxy-5-methoxybenzoic acid (12)

Table 5 ^{1}H NMR data of compound **7**, **8**, **9**, **10** and **11** (400 MHz, $CDCl_3$)

No.	7	8	9	10	11
3				3.93 (m)	3.53 tdd, (4.4, 4.0, 3.6)
4		5.82 (s)	6.17 (s)		
5					5.36 (t, 6.4)
6	5.66 (s)	4.34 (m)		6.20 (d, 8.4)	
7				6.46 (d, 8.4)	
18	0.65 (s)	0.75 (s)	0.72 (s)	0.77 (s)	
19	1.12 (s)	1.38 (s)	1.16 s)	0.84 (s)	0.93 (d, 6.4)
20					
21	0.85 (d, 6.0)	0.93 (d, 6.4)	0.93 (d, 6.4)	0.95 (d, 6.4)	
22				5.08 (m)	
23				5.12 (m)	
24					0.84 (t, 7.2)
25					
26	0.81 (d, 7.6)	0.84 (d, 6.8)	0.84 (d, 8.0)	0.77 (d, 6.8)	0.83 (d, 6.4)
27	0.74 (d, 6.4)	0.82 (d, 6.4)	0.82 (d, 7.6)	0.79 (d, 6.4)	0.81 (d, 6.4)
28				0.86 (d, 6.8)	0.68 (s)
29	0.77 (t, 7.6)	0.85 (t, 6.8)	0.85 (t, 7.6)		1.01 (s)

Compound 15 was obtained as colorless oil. Its IR spectrum showed the presence of hydroxyl groups at 3410 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **15** (Table 7) and comparison with the corresponding values reported in the literature,[16] the structure was assigned as I**sointermedeol**.



I**sointermedeol (15)**

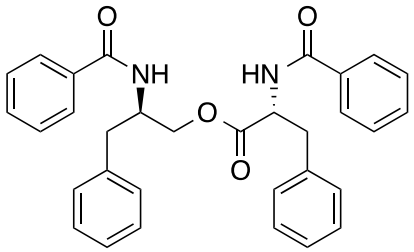
Table 6 ^{13}C NMR data of compound **7**, **8**, **9**, **10** and **11** (100 MHz, $CDCl_3$)

NO.	7	8	9	10	11
1	35.7	37.1	35.5	34.7	37.5
2	32.0	34.3	34.0	30.1	31.9
3	32.9	200.1	199.5	66.5	72.0
4	33.8	126.3	125.4	28.6	42.5
5	171.4	168.4	161.1	82.1	140.9
6	123.4	73.2	202.3	135.4	121.9
7	199.3	38.6	46.8	130.7	32.1
8	35.6	29.7	39.1	79.4	32.1
9	53.8	53.6	51.0	51.1	50.3
10	38.5	38.0	34.2	37.0	36.7
11	21.0	21.0	20.9	20.9	21.3
12	39.6	39.6	39.8	39.3	39.9
13	42.3	42.5	42.5	44.6	42.6
14	55.8	55.9	55.9	51.7	56.9
15	24.1	24.1	24.0	23.4	26.3
16	28.1	28.2	28.0	29.7	28.5
17	56.0	56.1	56.5	56.2	56.3
18	11.9	12.0	12.0	12.9	36.3
19	17.3	19.5	17.5	18.2	19.2
20	36.1	36.1	36.0	39.7	54.2
21	18.7	18.7	18.7	19.6	26.3
22	33.8	33.9	33.8	135.2	46.1
23	26.0	26.1	26.0	132.3	23.3
24	45.8	45.8	45.8	42.8	12.2
25	29.1	29.2	29.1	33.1	29.4
26	19.8	19.8	19.8	19.9	20.1
27	19.0	19.0	19.0	20.6	19.6
28	23.0	23.1	23.1	17.5	19.0
29	11.9	12.0	11.9		12.0

Table 7 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of compound **12** and **15**

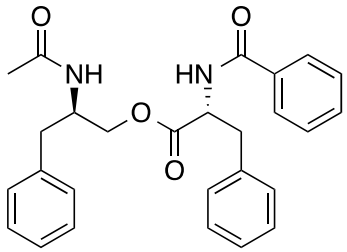
	12 ($CDCl_3$+CD_3OD)		**15** ($CDCl_3$)	
No.	δ_H	δ_C	δ_H	δ_C
1		121.9	1.34 (m), 1.06 (m)	41.4
2		150.5	1.50 (m)	20.1
3	6.83 (d, 8.4)	112.4	1.77 (m), 1.33 (m)	43.5
4	7.56 (dd, 8.4, 1.6)	124.4		72.0
5		146.6	1.32 (m)	49.1
6	7.48 (d, 2.0)	114.3	2.04 (dd, 13.6, 2.0), 1.49 (m)	22.7
7		168.8	2.41 (brs)	39.3
8			1.80 (m)	23.5
9			1.38 (d, 4.4), 1.11 (m)	40.3
10				35.3
11				146.9
12			4.90 (brs), 4.86 (brs)	110.8
13			1.76 (s)	22.8
14			0.92 (s)	18.4
15			1.09 (s)	22.3
5-OCH_3	3.86 (s)	55.9		

Compound 13 was obtained as white solid. The IR spectrum exhibited characteristic bands at 3304 and 1637 cm^{-1} (NH-CO), 1750 and 1217 cm^{-1} (CO-OR), 747 and 695 cm^{-1} (unsubstituted phenyl). Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **13** and comparison with the corresponding values reported in the literature,[15] the structure was assigned as **(-)-Auranamide.**



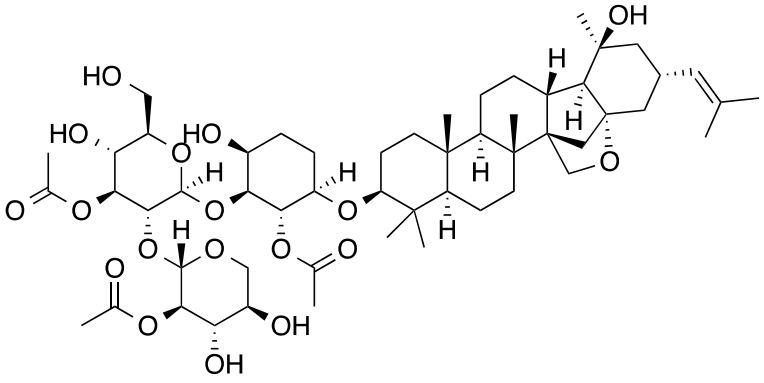
(-)-Auranamide (13)

Compound 14 was obtained as white solid (m.p. 188-189 $^{\circ}$C). The IR spectrum exhibited characteristic bands at 3318 and 1641 cm^{-1} (NH-CO), 1720 and 1233 cm^{-1} (CO-OR), 741 and 695 cm^{-1} (unsubstituted phenyl). Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **14** and comparison with the corresponding values reported in the literature,[15] the structure was assigned as D-phenylalanine, ***N*-Benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester.**



***N*-Benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester (14)**

Compound 16 was obtained as a amorphous solid. Its IR spectrum showed the presence of hydroxyl groups at 3388 cm^{-1} and acetyl group at 1733 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **16** (Table 8) and comparison with the corresponding values reported in the literature,[17] the structure was assigned as **3",2'"-*O*-acetylcolubrin**.

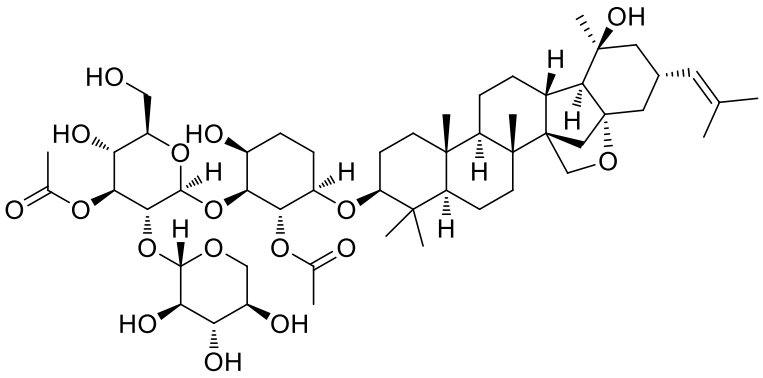


3",2'"-*O*-acetylcolubrin (16)

Table 8 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of compound **16** and **19** ($CDCl_3$+CD_3OD)

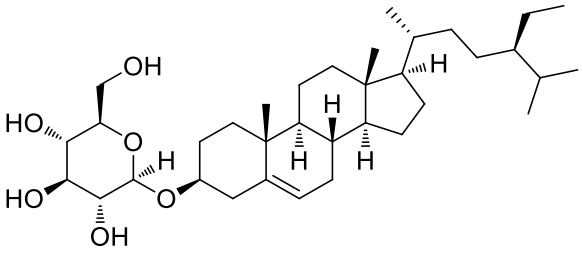
16			**19**		
No.	δ_H	δ_C	No.	δ_H	δ_C
1	1.59 (m)	38.4			
2	1.77 (m)	25.8			
3	2.97 (m)	89.4			
4		39.0			
5	3.82 (m)	55.9			
6	1.46 (m)	17.9			
7	1.41 (m)	35.6			
8		37.0			
9	0.73 (m)	52.7			
10		37.2			
11	1.54 (m)	21.3			
12	1.77 (m)	27.9			
13	2.34 (m)	36.8			
14		53.3			
15	1.95 (m), 1.21 (m)	36.0			
16		109.8			
17	1.01(d, 6.0)	52.7			
18	1.04 (s)	18.4			
19	0.76 (s)	15.9			
20		68.6			
21	1.10 (s)	29.2			
22	1.41 (m)	44.2			
23	4.60 (m)	68.2			
24	5.12 (m)	124.9			
25		135.6			
26	1.62 (s)	18.4			
27	1.65 (s)	25.4			
28	0.84 (s)	27.5			
29	0.65 (s)	15.9			
30	3.88 (m)	65.8			
1′	4.24 (d, 8.0)	103.8	1′	4.22 (d, 7.8)	103.8
2′	5.10 (m)	70.7	2′	5.10 (m)	71.0
3′	3.66 (dt, 8.4, 4.0)	80.3	3′	3.69 (m)	79.6
4′	3.93 (m)	68.4	4′	3.96 (m)	68.3
5′	3.44 (d, 12.8)	65.5	5′	3.41 (m)	65.5
$OCOCH_3$-2′		170.4	$OCOCH_3$-2′		170.3
$OCOCH_3$-2′	2.05 (s)	21.2	$OCOCH_3$-2′	2.00 (s)	21.1
1″	4.47 (d, 7.2)	101.2	1″	4.45 (m)	101.2
2″	3.51 (m)	76.6	2″	3.46 (m)	76.2
3″	4.92 (t, 10.0)	77.1	3″	3.48 (m)	75.8
4″	3.51 (m)	63.9	4″	3.37 (m)	69.7
5″	3.24 (m)	75.9	5″	3.35 (m)	75.9
6″	3.72 (m)	60.7	6″	3.70 (m)	60.5
$OCOCH_3$-3″		170.9	$OCOCH_3$-3″		171.4
$OCOCH_3$-3″	2.05 (s)	20.6	$OCOCH_3$-3″	2.00 (s)	20.6
1‴	4.44 (d, 8.0)	101.3	1‴	4.46 (m)	103.8
2‴	4.58 (d, 9.6)	73.3	2‴	4.58 (m)	73.2
3‴	3.32 (m)	75.1	3‴	3.38 (m)	73.7
4‴	3.56 (m)	69.8	4‴	3.48 (m)	69.6
5‴	3.88 (m), 3.09 (t, 10.4)	65.5	5‴	4.32 (d, 10.0), 4.14	63.3
$OCOCH_3$-2‴		170.9	$OCOCH_3$-2‴	-	-
$OCOCH_3$-2‴	2.11 (s)	20.9	$OCOCH_3$-2‴	-	-

Compound 19 was obtained as a amorphous solid. Its IR spectrum showed the presence of hydroxyl groups at 3399 cm^{-1} and acetyl group at 1735 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **19** (Table 8) and comparison with the corresponding values reported in the literature,[17] the structure was assigned as **3"-*O*-Acetylcolubrin**.



3"-*O*-Acetylcolubrin (19)

Compound 20 was obtained as a amorphous solid. Its IR spectrum showed the presence of hydroxyl groups at 3368 cm^{-1}. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **20** (Table 9) and comparison with the corresponding values reported in the literature,[19] the structure was assigned as ***β*-Sitosterol-3-*O*-glycoside**.

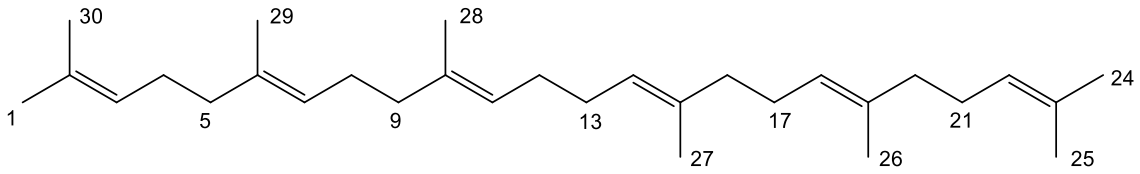


***β*-Sitosterol-3-*O*-glycoside (20)**

Table 9 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra data of compound **20** ($CDCl_3$+CD_3OD)

	20	
No.	δ_H	δ_C
1	1.38 (m)1.13 (m)	30.4
2	1.92 (m), 1.59 (m)	29.9
3	3.55 (m)	78.9
4	2.35 (m) 2.20 (m)	38.5
5	-	140.3
6	5.33 (bs)	122.0
7	1.96 (m), 1.80 (m)	30.1
8	1.45 (m)	31.9
9	1.44 (m)	50.3
10	-	31.8
11	1.52 (m), 1.27 (m)	22.7
12	1.49 (m), 1.24 (m)	37.2
13	-	40.0
14	1.25 (m)	56.0
15	1.60 (m), 1.35 (m)	27.7
16	1.60 (m), 1.35 (m)	27.3
17	1.40 (m)	56.7
18	0.80 (s)	19.0
19	0.97 (s)	19.4
20	1.64 (m)	36.1
21	1.06 (m)	18.5
22	1.25 (m)	33.9
23	1.25 (m)	29.9
24	1.46 (m)	46.1
25	1.82 (m)	31.7
26	0.89 (d, 7.8)	18.7
27	0.88 (d, 7.8)	18.7
28	1.29 (m)	25.8
29	0.65 (s)	11.6
1′	4.37 (d, 7.8)	101.1
2′	3.15 (dd, 7.8, 8.3)	73.5
3′	3.30 (bs)	75.9
4′	3.35 (m)	70.1
5′	3.24 (bs)	76.5
6′	3.79 (d, 11.2), 3.67 (dd, 11.2, 4.4)	61.6

Compound 21 was obtained as a amorphous solid. The IR spectrum showed the intense bands at 3014, 2976, 2937, 2919 and 2850 cm^{-1} were characterized as aliphatic C-H strtching, while its corresponded bending vibrations appeared at 1453 and 1377 cm^{-1}. The ^{1}H and ^{13}C NMR spectra (Table 10) showed the characteristic signal pattern of a terpenoid unit. Based on analysis of the one- and two-dimensional ^{1}H and ^{13}C NMR data of compound **21** and comparison with the corresponding values reported in the literature,[18] the structure was assigned as **Squalene**.



Squalene (21)

Table 10 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra data (100 MHz) of compound **21** ($CDCl_3$)

No.	21		No.		
	δ_H	δ_C		δ_H	δ_C
1	1.68 s	25.6	16	1,98 m	39.7
2		131.2	17	2.09 m	26.8
3	5.15 m	124.4	18	5.11 m	124.3
4	2.09 m	26.7	19		134.9
5	1.98 m	39.7	20	1.98 m	39.7
6		134.9	21	2.09 m	26.7
7	5.11 m	124.3	22	5.15 m	124.4
8	2.09 m	26.8	23		131.2
9	1.98 m	39.7	24	1.68 s	25.6
10		135.1	25	1.60 s	16.0
11	5.11 m	124.3	26	1.60 s	16.0
12	1.98 m	28.3	27	1.60 s	16.0
13	1.98 m	28.3	28	1.60 s	16.0
14	5.11 m	124.3	29	1.60 s	16.0
15		135.1	30	1.60 s	16.0

Physical properties and spectroscopic data

Ceanothic acid (**1**): white solid; 354-356 ^{O}C; R_f = 0.49 (5%MeOH/45%EtOAc/hexane); IR (neat) ν_{max} (cm^{-1}): 3330, 3066, 2935, 2869, 2729, 2622, 1685, 1454, 1404, 1316, 1221, 1022, 754; ^{1}H and ^{13}C NMR data were given in Table 2 and 3, respectively.

Granulosic acid (**2**): white solid; 237-239 ^{o}C; R_f = 0.49 (5%MeOH/45%EtOAc/hexane); IR (neat) ν_{max} (cm^{-1}): 3410, 2972, 2932, 1714, 1453, 1376, 1164, 1074,754; ^{1}H and ^{13}C NMR data were given in Table 2 and 3, respectively.

Zizyberenalic acid (**3**) : white solid; 216-214 ^{o}C; R_f = 0.63 (40%EtOAc/hexane); IR (neat) ν_{max} (cm^{-1}): 3362, 3071, 2942, 2871, 1695, 1456, 1380, 1203, 1022, 886, 756; ^{1}H and ^{13}C NMR data were given in Table 2 and 3, respectively.

Colubrinic acid (**4**): white solid; 262-264 ^{o}C; R_f = 0.50 (5%MeOH/CH_2Cl_2); IR (neat) ν_{max} (cm^{-1}): 3432, 2922, 2852, 1707, 1461, 1262, 750; ^{1}H and ^{13}C NMR data were given in Table 2 and 3, respectively.

Alphitolic acid (**5**): white solid; 232-234 ^{o}C; R_f = 0.46 (40%EtOAc/hexane); IR (neat) ν_{max} (cm^{-1}): 3362, 2938, 2866, 1697, 1641, 1455, 1378, 1222, 1184, 1039, 883, 757; ^{1}H and ^{13}C NMR data were given in Table 4.

Betulinic acid (**6**): white solid; 304-306 ^{o}C; R_f = 0.49 (80%EtOAc/hexane); IR (neat) ν_{max} (cm^{-1}): 3400, 2938, 2866, 1697, 1455, 1377, 1272, 1224, 1183, 1109, 1029, 981, 885, 756; ^{1}H and ^{13}C NMR data were given in Table 4.

Stigmast-5-en-7-one (**7**): amorphous solid; R_f = 0.41 (85%CH_2Cl_2/hexane); IR (neat) ν_{max} (cm^{-1}): 2934, 2868, 1715, 1673, 1616, 1461, 1378, 1331, 1269, 1230, 1186, 1126, 1022, 932, 865, 748; ^{1}H and ^{13}C NMR data were given in Table 5 and 6, respectively.

6β-Hydroxystigmast-4-en-3-one (**8**): white solid; 192-194 ^{o}C; R_f = 0.53 (5%MeOH/CH_2Cl_2); IR (neat) ν_{max} (cm^{-1}): 3503, 2952, 2869, 1733, 1690, 1615, 1464, 1382, 1265, 1244, 1192, 1151, 1126, 1088, 1037, 1015, 965, 921, 876, 832, 795, 739, 704; ^{1}H and ^{13}C NMR data were given in Table 5 and 6, respectively.

Stigmast-4-ene-3,6-dione (**9**): white solid; 304-306 ^{o}C; R_f = 0.49 (80%EtOAc/hexane); IR (neat) ν_{max} (cm^{-1}): 3144, 2954, 2869, 1687, 1605, 1463, 1414, 1379, 1327, 1263, 1219, 1193, 1175, 1120, 1084, 1020, 1000, 948, 924, 869, 751, 679, 636; ^{1}H and ^{13}C NMR data were given in Table 5 and 6, respectively.

Ergosterol peroxide (**10**): white solid; 180-182 ^{o}C; R_f = 0.34 (30%EtOAc/hexane); IR (neat) ν_{max} (cm^{-1}): 3516, 3296, 2953, 2870, 1703, 1655, 1456, 1376, 1299, 1264, 1226, 1172, 1151, 1107, 1075, 1044, 1026, 966, 934, 857, 818, 777, 724, 653, 631; ^{1}H and ^{13}C NMR data were given in Table 5 and 6, respectively.

β-Sitosterol (**11**): white solid; 143-144 ^{o}C; R_f = 0.49 (80%EtOAc/hexane); IR (neat) ν_{max}(cm^{-1}): 3428, 2936, 2867, 2849, 1666, 1464, 1381, 1107, 1050 1021, 958, 800; ^{1}H and ^{13}C NMR data were given in Table 5 and 6, respectively.

2-Hydroxy-5-methoxybenzoic acid (**12**): white solid; 145-146 ^{o}C; R_f = 0.52 (40%acetone/hexane); IR (neat) ν_{max} (cm^{-1}): 3482, 3094, 2946, 2834, 2647, 2566, 1677, 1597, 1521, 1469, 1455, 1432, 1381, 1282, 1237, 1205, 1112, 1027, 916, 882, 803, 761, 735, 635; ^{1}H and ^{13}C NMR data were given in Table 7.

(-)-Auranamide (**13**): white solid; 207-209 ^{o}C; R_f = 0.58 (15%EtOAc/CH_2Cl_2); IR (neat) ν_{max} (cm^{-1}): 3304, 3060, 3028, 2917, 2849, 1750, 1705, 1637, 1603, 1578, 1530, 1490, 1457, 1389, 1354, 1326, 1304, 1272, 1217, 1100, 1078, 1030, 1000, 921, 872, 853, 827, 799, 747, 722, 695; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.70-7.22 (m, 20H, ArH), 6.76 (brd,1H, J = 8.4 Hz, NH-10), 6.69 (brd, 1H, J = 4.8 Hz, NH-7), 4.92 (q, 1H, J = 6.4 Hz, H-2), 4.62 (m, 1H, H-5), [4.40 (dd, 1H, J = 11.2, 2.8 Hz, H-4), 4.02 (dd, 2H, J = 11.2, 4.4 Hz, H-4)], [3.29, 3.21 (dddd, 2H, J = 14.0, 6.0 Hz, H-1)], [3.00, 2.89 (dddd, 2H, J = 14.0, 6.0 Hz, H-6)]; ^{13}C NMR: δ 171.9, 168.4, 168.2, 137.1, 135.8, 134.2, 133.3, 132.0,

131.4, 129.3 x 2, 129.2 x 2, 128.8 x2, 128.7 x 2, 128.6 x 2, 128.4 x 2, 128.0, 127.3 x 2, 127.1 x 2, 126.8, 66.4, 55.5, 51.3, 37.5, 37.2.

D-Phenylalanine, *N*-benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester (**14**): white solid; 188-189 ^{o}C; R_f = 0.38 (15%EtOAc/CH_2Cl_2); IR (neat) ν_{max} (cm^{-1}): 3318, 2926, 2856, 2355, 2326, 1720, 1641, 1576, 1537, 1455, 1378, 1264, 1233, 1179, 1040, 982, 741, 701; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72-7.06 (m, 15H, ArH), 6.83 (brd,1H, *J* = 7.6 Hz, NH-10), 6.04 (brd, 1H, *J* = 8.8 Hz, NH-7), 6.77 (dd, 1H, *J* = 14.0, 8.0 Hz, H-2), 4.34 (m, 1H, H-5), [3.93, 3.81 (dddd, 2H, *J* = 12.0, 4.8 Hz, H-6)], [3.21, 3.06 (dddd, 2H, *J* = 12.0, 6.0 Hz, H-1)], 2.75 (m, 2H, H-4), 2.02 (s, 3H, CH_3)]; ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.8, 170.3, 167.1, 136.7, 136.6, 133.6, 131.9, 129.3 x 2, 129.1 x 2, 128.7 x 2, 128.6 x 2, 128.5 x 2, 127.1, 127.0 x 2, 126.7, 64.6, 55.0, 49.4, 38.4, 37.4, 20.8.

Isointermedeol (**15**): colorless oil; R_f = 0.48 (85%CH_2Cl_2/hexane); IR (neat) ν_{max} (cm^{-1}): 3410, 3014, 2975, 2919, 2850, 1715, 1452, 1377, 1215, 1163, 1074, 1048, 750, 667; ^{1}H and ^{13}C NMR data were given in Table 7.

3",2"'-*O*-Acetylcolubrin (**16**): amorphous solid; R_f = 0.48 (40%MeOH/CH_2Cl_2); IR (neat) ν_{max} (cm^{-1}): 3388, 2939, 2868, 1736, 1648, 1452, 1373, 1238, 1029, 751; ^{1}H and ^{13}C NMR data were given in Table 8.

3β-Benzoylcolubenzoylatic acid (**17**): white solids; mp 160-162 ^{o}C; $[\alpha]_D^{25}$ -14.3 (*c* 0.1, CH_2Cl_2); R_f: 0.49 (EtOAc-*n*-hexane, 2:3); IR (neat) ν_{max} (cm^{-1}): 3070, 2942, 2866, 1719, 1453, 1374, 1271, 1171, 1107, 1027, 976, 947, 886, 756, 712; HRESITOFMS: *m/z* 747.3865 $[M + Na]^+$ (calcd for $C_{45}H_{56}O_8$ + Na, 747.3867); ^{1}H and ^{13}C NMR data were given in Table 1.

3β-Acetylcolubenzoylatic acid **18**: white solids; mp 96-98 ^{o}C; $[\alpha]_D^{25}$ -4.2 (*c* 0.1, CH_2Cl_2); R_f: 0.55 (EtOAc-*n*-hexane, 2:3); IR (neat) ν_{max} (cm^{-1}): 2933, 2862, 1722, 1454, 1371, 1270, 1239, 1171, 1124, 1027, 759, 714; HRESITOFMS: *m/z* 685.3710 $[M + Na]^+$ (calcd for $C_{40}H_{54}O_8$ + Na, 685.3711) ^{1}H and ^{13}C NMR data were given in Table 1.

3''-*O*-Acetylcolubrin (**19**): amorphous solid; R_f = 0.40 (40%MeOH/CH_2Cl_2); IR (neat) ν_{max} (cm^{-1}): 3399, 2938, 2867, 1733, 1448, 1373, 1241, 1038, 752; ^1H and ^{13}C NMR data were given in Table 8.

β-Sitosterol-3-*O*-glycoside (**20**): amorphous solid; R_f = 0.41 (5%MeOH/50%EtOAc/ hexane); IR (neat) ν_{max} (cm^{-1}): 3368, 2941, 1664, 1512, 1457, 1375, 1246, 1026, 751; ^1H and ^{13}C NMR data were given in Table 9.

Squalene (**21**): yellow oil; R_f= 0.64 (10% CH_2Cl_2:hexane); IR (neat) ν_{max} (cm^{-1}): 3014, 2976, 2919, 2850, 1715, 1453, 1377, 1216, 1164, 1074, 1049; ^1H and ^{13}C NMR data were given in Table 10**.**

Conclusion

The branches and roots extracts of *C. asiatica* were investigated and led to the isolation isolation of twenty-one compounds. The crude extracts from the branches of *C. asiatica* gave sixteen compounds, including six triterpene acids: ceanothic acid (**1**), granulosic acid (**2**), zizyberenalic acid (**3**), colubrinic acid (**4**), alphitolic acid (**5**) and betulinic acid (**6**); six steroids: stigmast-5-en-7-one (**7**), 6β-hydroxystigmast-4-en-3-one (**8**), stigmast-4-ene-3,6-dione (**9**), ergosterol peroxide (**10**), β-sitosterol (**11**) and β-sitosterol-3-*O*-glycoside (**20**); 2-hydroxy-5-methoxybenzoic acid (**12**); two phenylanalines: (-)-auranamide (**13**) and D-phenylalanine, *N*-benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester (**14**); sesquiterpenoid: isointermedeol (**15**); two jujubogenin glycosides: 3'',2'''-*O*-acetylcolubrin (**16**). The crude extracts from the roots of *C. asiatica* yielded two new ceanothane triterpenes, 3β-benzoylcolubenzoylatic acid (**17**) and 3β- acetylcolubenzoylatic acid (**18**), along with nine known compounds, including five triterpene acids: compound **1**-**3** and **5**-**6**; steroid: β-sitosterol-3-*O*-glycoside (**20**); two jujubogenin glycosides: 3'',2'''-*O*-acetylcolubrin (**16**) and 3''-*O*-acetylcolubrin (**19**); triterpenoid: squalene (**21**). Structures of the isolated compounds were identified from spectroscopic evidence. Compounds **3**, **10**, **17**, **18** and **21** showed antimalarial activity against *Plasmodium falciparum* with IC_{50} values ranging from 2.32 to 4.67 µg/mL, while compounds **5** and **18** showed antimycobacterial activity against *Mycobacterium tuberculosis* (MIC 50.00 and 6.25 µg/mL, respectively). In addition, compounds **3**, **5**, **6**, **10**, **14**, **16**-**19** and **21** showed cytotoxicity against cancer cell lines, KB, NCI-H187 and MCF-7 with IC_{50} values ranging from 8.32 to 46.72 µg/mL. Compounds **2**, **3** and **7**-**15** are reported

from the genus *Colubrina* for the first time. Moreover, compounds **2**, **3**, **7-9** and **12-13** are reported for the first time in the family Rhamnaceae.

Table 11 Biological activities from the branches and roots of *C. asiatica*

Compound	antimalarial	antimycobacterial	cytotoxicity (IC_{50}, μg/mL)		
	(IC_{50}, μg/mL)	(MIC, μg/mL)	KB[a]	NCI-H187[b]	MCF-7[c]
1	inactive	inactive	inactive	inactive	inactive
2	inactive	inactive	inactive	inactive	inactive
3	4.18	inactive	18.17	15.46	inactive
4	inactive	nd[d]	inactive	inactive	inactive
5	inactive	50.00	32.09	17.57	18.42
6	inactive	inactive	18.06	16.52	inactive
7	inactive	nd[d]	inactive	inactive	inactive
8	inactive	nd[d]	inactive	inactive	inactive
9	inactive	nd[d]	inactive	inactive	inactive
10	3.08	nd[d]	29.48	19.30	inactive
11	inactive	nd[d]	inactive	inactive	inactive
12	inactive	nd[d]	inactive	inactive	inactive
13	inactive	nd[d]	inactive	inactive	inactive
14	inactive	nd[d]	inactive	19.51	inactive
15	inactive	nd[d]	inactive	inactive	inactive
16	inactive	inactive	37.46	nd[d]	41.83
17	4.67	inactive	18.47	8.32	inactive
18	3.07	6.25	35.44	11.31	inactive
19	inactive	inactive	44.11	nd[d]	46.72
20	inactive	inactive	inactive	nd[d]	inactive
21	2.32	inactive	inactive	15.50	46.19
dihydroartemisinin	0.002				
isoniazid		0.047			
ellipticine			1.21	0.935	
tamoxifen					7.00
doxorubicin			0.855	0.057	7.90

[a]Human epidermoid carcinoma in the mouth, [b]Human lung cancer cell,
[c]Human breast cancer cell, [d]Not determined.

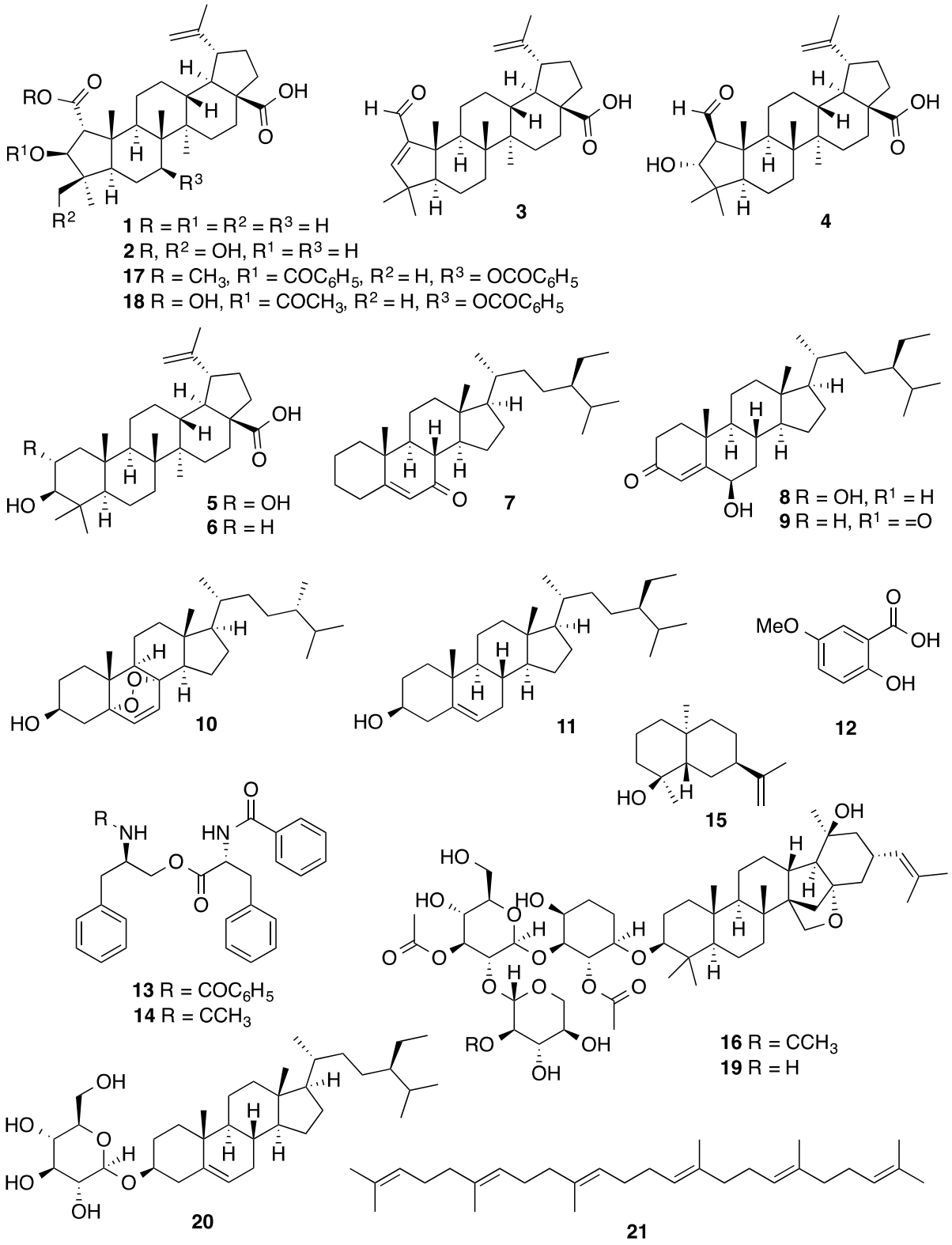


Figure 12 Structures of compounds **1-21**

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OUTPUT OF THE RESEARCH

MSc. Students:

Mr. Watchara Sangsopha (2014-2016): “Chemical constituents and biological activities from aerial parts of *Sphaeranthus indicus*” Master of Science Thesis in Organic Chemistry, Graduate School, Khon Kaen University.

Publication papers:

1. Sangsopha, W., Kanokmedhakul, S., Lekphrom R., Kanokmedhakul, K. Chemical constituents and biological activities from branches of *Colubrina asiatica*. Natural Product Research DOI: 10.1080/14786419.2017.1320787. (IF = 1.057)
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APPENDIX

NATURAL PRODUCT RESEARCH, 2017
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SHORT COMMUNICATION

Chemical constituents and biological activities from branches of *Colubrina asiatica*

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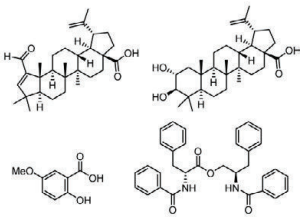
ABSTRACT

Sixteen compounds were isolated from a Thai medicinal plant, *Colubrina asiatica*. The isolated compounds were elucidated on the basis of spectroscopic methods (IR, 1D and 2D NMR) as six triterpene acids (**1**–**6**), five steroids (**7**–**11**), one benzoic acid derivative (**12**), two peptides (**13** and **14**), one sesquiterpenoid (**15**) and one jujubogenin (**16**). Compounds **3** and **10** showed antimalarial activity against *Plasmodium falciparum*. Compound **5** showed antimycobacterial activity. Moreover, compounds **3**, **5**, **6**, **10** and **14** exhibited weak cytotoxicity against cancer cell lines. Compounds **1**–**15** have been isolated for the first time from this plant.



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Colubrina asiatica; triterpene acids; steroids; antimalarial; antimycobacterial; cytotoxicity

1. Introduction

Colubrina asiatica (L.) Brongn (Rhamnaceae) is a rambling shrub, 4 m in height, found widely in South-East Asia, tropical Australia and in the Pacific Islands. In Thailand, it is known as 'Khan song' or 'Kan thoeng'. The leaves and bark are used traditionally as a decoction for the treatment of skin diseases and the roots as a cure for fever and thirst (Phonsena 2007). Previous reports of compounds from the leaves of *C. asiatica* and related *Colubrina* species led to the isolation of saponins (Wagner et al. 1983; Seaforth et al. 1992; Oulad-Ali et al. 1994; Lee et al. 2000; Li et al. 1999; ElSohly et al. 1999), triterpenes (Roitman & Jurd 1978; Baxter & Walkinshaw 1988) as well as alkaloids and phenolic compounds (Guinaudeau et al. 1976).

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As part of our research on Thai medicinal plants containing potential bioactive compounds, air-dried branches of *C. asiatica* were investigated. Both EtOAc and MeOH extracts showed activity against *Plasmodium falciparum*, *Mycobacterium tuberculosis* and cytotoxicity towards three cancer cell lines. We report herein the first isolation, characterisation and biological activities of 16 isolated compounds from the branches of this plant.

2. Results and discussion

Sixteen isolated compounds from branches of *C. asiatica* were identified by physical and spectroscopic methods (IR, 1D and 2D NMR) as well as by comparison with the spectral data of known compounds reported in literature. They consist of six triterpene acids: ceanothic acid (**1**), granulosic acid (**2**) (Roitman & Jurd 1978), zizyberenalic acid (**3**) (Kundu et al. 1989), colubrinic acid (**4**) (Baxter & Walkinshaw 1988), alphitolic acid (**5**) (Baxter & Walkinshaw 1988) and betulinic acid (**6**) (Kundu et al. 1989); five steroids: stigmast-5-en-7-one (**7**) ang6*β*-hydroxystigmast-4-en-3-one (**8**) (Aguilar-Gonzalez et al. 2005), stigmast-4-ene-3,6-dione (**9**) (Ghosh & Bhattacharya 2005), ergosterol peroxide (**10**) (Shin et al. 2001) and *β*-sitosterol (**11**) (Qiao et al. 1999); 2-hydroxy-5-methoxybenzoic acid (**12**) (Al-Rawi & Jasim 1982); two phenylalanine derivatives: (-)-auranamide (**13**) and D-phenylalanine, *N*-benzoyl-(2*R*)-2-(acetylamino)-3-phenylpropyl ester (**14**) (Jakupovic et al. 1987); a sesquiterpenoid: isointermedeol (**15**) (Thappa et al. 1979); and a jujubogenin glycoside: 3″-*O*-acetylcolubrin (**16**) (Lee et al. 2000) (Figure 1 and supplementary material).

Zizyberenalic acid (**3**) and ergosterol peroxide (**10**) showed antimalarial activity against *P. falciparum* with IC_{50} values of 4.18 and 3.08 µg/mL, respectively, which agree well with previous reported values (Suksamrarn et al. 2006; Phonkerd et al. 2008). Among isolated compounds, only alphitolic acid (**5**) showed antimycobacterial activity against *M. tuberculosis*, with MIC value of 50.00 µg/mL which supported the previous reported value (Suksamrarn et al. 2006). This is the first report for cytotoxicity of **3** against KB and NCI-H187 cell lines with IC_{50} values of 18.17 and 15.46 µg/mL, respectively. Compound **5** showed cytotoxicity against KB, NCI-H187 and MCF-7 with IC_{50} of 32.09, 17.57 and 18.42 µg/mL, respectively. Betulinic acid (**6**) has been reviewed with a wide range of cytotoxicity against human cancer cell lines (Periasamy et al. 2014). However, this is the first report for its cytotoxicity against NCI-H-187 (IC_{50} = 16.52 µg/mL). Betulinic acid (**6**) also exhibited cytotoxicity against KB cell lines (IC_{50} = 18.06 µg/mL) which is comparable to the previously reported value (Jeong et al. 1999). Moreover, cytotoxicity values of **10** against KB (IC_{50} = 29.48 µg/mL) and NCI-H187 (IC_{50} = 19.30 µg/mL) corresponded to previously reported values (Prompiboon et al. 2008 and Kornsakulkarn et al. 2016). In addition, this is the first report for cytotoxicity of compound **14** against NCI-H-187 (IC_{50} = 19.51 µg/mL). Due to the general criteria, cytotoxicity of compound is IC_{50} < 10 µg/mL. Therefore, antiplasmodial and antimycobacterial activities of isolated compounds were not due to their general cytotoxicity. However, all bioactive compounds showed weak activities comparing to their reference drugs. The result of biological activities of isolated compounds is shown in Table S1 in supplementary material.

3. Conclusion

The extracts of *C. asiatica* branches were investigated and led to the isolation of 16 known compounds, including six pentacyclic triterpene acids, five steroids (**7**–**11**), 2-hydroxy-5-methoxybenzoic acid (**12**), two peptides (**13** and **14**), intermedeol (**15**) and 3″-*O*-acetylcolubrin

(**16**). Compounds **2**, **3** and **7**–**15** are reported from the genus *Colubrina* for the first time. Moreover, compounds **2**, **3**, **7**–**9** and **12**–**13** are reported for the first time in the family Rhamnaceae. Several of isolated compounds exhibited antimalarial activity and cytotoxicity towards KB, NCI-H187 and MCF-7 cancer cell lines.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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A new coumarin from the roots of *Micromelum minutum*

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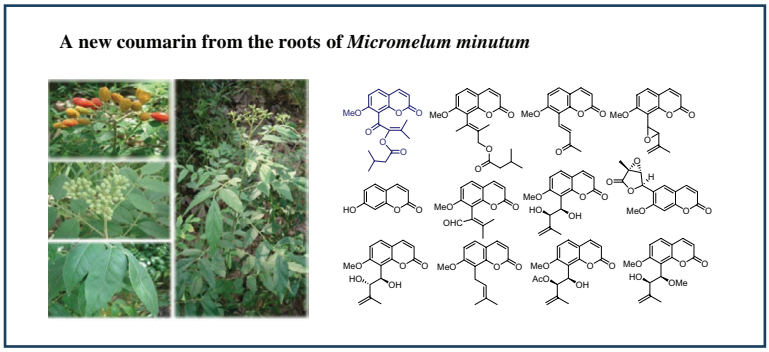
ABSTRACT

A new coumarin, minutuminolate (**1**), together with eleven known coumarins (**2**–**12**), was isolated from the roots of *Micromelum minutum*. The structures of these compounds were established on the basis of their 1D and 2D NMR spectroscopic data. Compounds **2**, **5**, **10**, **11** and **12** showed cytotoxicity against KB cell line. In addition, compounds **2**, **3**, **4**, **7**, **11** and **12** also showed weak cytotoxicity against NCI-H187 cell line.



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1. Introduction

The genus *Micromelum* belongs to the flowering plants in the Rutaceae family. *Micromelum minutum* (Forst. F.) Wight & Arn (syn. *M. pubescens* Blume), or 'Hat-Sa Khun' in Thai, is a tropical small spineless tree that can reach up to 3 m in height. It is widely distributed in Southeast Asia and in Pacific islands. The stems, flowers, leaves and roots are used medicinally for a variety of indications (Pongboonrod 1958). A water decoction of the roots is traditionally used for treatment of fever, giddiness and haemorrhoids (Bunyapraphatsara & Chokechaijaroenporn 2000). Previous phytochemical studies on the genus *Micromelum* have revealed a number of coumarins, particularly the 6- and 8-prenylated 7-methoxycoumarins (Lamberton et al. 1967; Cassady et al. 1979; Das et al. 1984; Kamperdick et al. 1999), polyoxygenated flavonoids, triterpenoids (Tantishaiyakul et al. 1986; Rahmani et al. 2003; Susidarti et al. 2006), quinolone alkaloid (Tantivatana et al. 1983) and carbazole alkaloids (Siridechakorn

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et al. 2012). In addition, we have previously reported C-7-oxygenated coumarins from the fruits of *M. minutum* (Lekphrom et al. 2011). Our continuous efforts to the phytochemistry research on the roots of this plant led to the isolation of a new coumarin (**1**) and eleven known coumarins (**2**–**12**) (Figure 1). In this paper, we describe the isolation, structural elucidation and biological activities of these compounds.

2. Results and discussion

The structures of the known compounds were identified by physical and spectroscopic data measurements ($[\alpha]_D$, ^{1}H and ^{13}C NMR, 2D NMR and MS) and compared to values from literature as a murralonginol isovalerate (**2**) (Ito et al. 1990), osthol (**3**) (Ito et al. 1990; Rashid et al. 1992), phebalosin (**4**) (Ito & Furukawa 1987; Rashid et al. 1992; Jiwajinda et al. 2000), micromelin (**5**) (Cassady et al. 1979), murrangatin acetate (**6**) (Ito & Furukawa 1987; Imai et al. 1989; Quader et al. 1992), osthenon (**7**) (Ito & Furukawa 1987), murrangatin (**8**) (Ito et al. 1990), minumicrolin (**9**) (Ito et al. 1990), murralongin (**10**) (Imai et al. 1986), umbelliferone (**11**) (De Silva et al. 1983) and murracarpin (**12**) (Wu et al. 1989; Lin & Wu, 1994).

Compound 1 was isolated as a colourless oil with a molecular formula of $C_{20}H_{22}O_6$ determined by the HRESITOFMS (*m/z* 381.1318 $[M + Na]^+$). The IR spectrum showed a conjugated lactone group at 1727 cm^{-1}. The UV spectrum showed maximal absorptions at 324 and 260 nm due to the 7-oxygenated coumarin (Lee & Soine 1969). The presence of a 7-methoxy-8-substituted coumarin skeleton was indicated by two pairs of doublets at δ 6.25 and 7.62 (1H each, d, J = 9.4 Hz) and at δ 7.44 and 6.85 (1H each, d, J = 8.6 Hz) in the ^{1}H NMR spectrum due to H-3 and H-4, H-5 and H-6, respectively. The remaining part of the unit

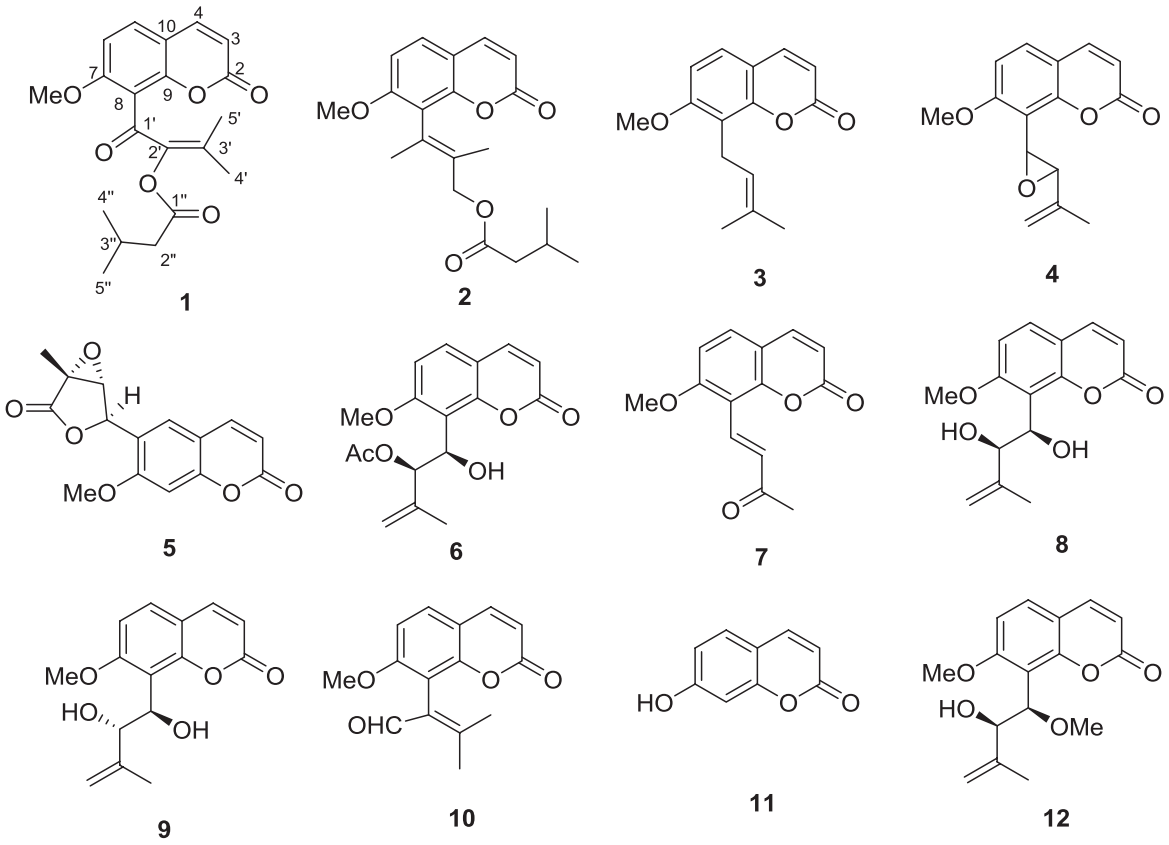

Figure 1. Structures of isolated compounds from the roots of *M. minutum*.

connected to C-8 was deduced from NMR spectral data to be a $C_{10}H_{15}O_3$ side chain. The ^{1}H NMR spectrum showed the presence of an isovaleryl group at δ 0.72 (6H, d, J = 6.8 Hz), 1.77 (1H, m) and methylene protons at δ 1.92 (2H, d, J = 6.8 Hz), and a 1,1-dimethyl-3-one-2-propenyl group at δ 1.82 (s, CH_3-4′), and 2.27 (s, CH_3-5′). It was found that the chemical shift of CH_3-5′ protons presented lower field than those of CH_3-4′ suggesting the anisotropic effect from the benzene ring. The ^{13}C NMR spectrum showed the presence of four methyl carbons at δ 20.4, 21.6 and 22.2 (overlapping), one methylene carbon at δ 42.4, one methine carbon at δ 25.1 and four quaternary carbons at δ 140.7, 142.7, 170.7 and 186.4 for the side chain. From these spectra data, the side chain at C-8 was elucidated as –CO(C=C(CH_3)$_2$)OCOR (R = isovaleryl). The COSY spectrum of **1** showed the connection of four spin systems: H-3/H-4, H-5/H-6, H-4′/H-5′ and H-2″/H-3″/H-4″/H-5″. The HMBC spectrum supported the structure of **1** by showing correlations of H-3 to C-2 and C-10; H-4 to C-2 and C-9; H-5 to C-7 and C-9; H-6 to C-8 and C-10; 7-OMe to C-7. Also, the HMBC correlations of H-4′ to C-1′, C-2′, C-3′ and C-5′; H-5′ to C-1′, C-2′, C-3′ and C-4′; H-2″ to C-1″, C-3″, C-4″ and C-5′″; H-4″ to C-2″, C-3″ and C-5″; H-5″ to C-2″, C-3″ and C-4″ (Figure S1) revealed that the 3-methyl-2-*O*-isovaleryl-1-oxo-butenyl unit was connected to C-8 of the coumarin skeleton. Compound **1** was thus identified as a new 7-methoxy-8-(3-methyl-2-*O*-isovaleryl-1-oxo-butenyl)-coumarin and has been named minutuminolate.

In the present investigation, all isolated compounds were tested for their bioactivities (Table 1). Compounds **2**, **3**, **5**, **11** and **12** showed cytotoxicity against KB cell line with IC_{50} values of 30.4, 19.9, 31.5 42.0 and 23.1 µg/mL, respectively. In addition, compounds **2**, **3**, **4**, **7**, **11** and **12** also showed cytotoxicity against NCI-H187 cell line with IC_{50} values of 49.5, 21.2, 16.6, 22.7, 19.4 and 20.2 µg/mL, respectively. While, only **2** showed cytotoxicity against MCF-7 cell line with an IC_{50} value of 25.4 µg/mL.

3. Experimental

3.1. General experimental procedures

Melting points were determined using an Electrothermal IA9200 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK). Optical rotations were measured on a

Table 1. Biological activities of the isolated compounds.

	Cytotoxicity (IC_{50}, µg/mL)		
Compound	KB[a]	NCI-H187[b]	MCF-7[c]
1	Inactive	Inactive	Inactive
2	30.4	49.5	25.4
3	19.9	21.2	Inactive
4	Inactive	16.6	Inactive
5	31.5	Inactive	Inactive
6	Inactive	Inactive	Inactive
7	Inactive	22.7	Inactive
8	Inactive	Inactive	Inactive
9	Inactive	Inactive	Inactive
10	Inactive	Inactive	Inactive
11	42.9	19.4	Inactive
12	23.1	20.2	Inactive
Ellipticine	0.50	0.43	0.32
Doxorubicin	0.14	0.32	0.26

[a]Human epidermoid carcinoma in the mouth.
[b]Human lung cancer cells.
[c]Human breast cancer cells.

JASCODIP-1000 digital polarimeter (JASCO Inc., USA), and UV spectra were recorded using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). IR spectra were obtained using a Bruker Tenser 27 spectrophotometer (Bruker, Germany). NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer (Varian Inc., USA) using $CDCl_3$, CD_3OD and DMSO-d_6 as solvents. The internal standards were referenced from the residue of those solvents. The HR-ESI-TOF-MS were recorded on a Bruker micrOTOF mass spectrometer (Brucker, Germany). Column chromatography (CC) was carried out on MERCK silica gel 60 (230–400 mesh) (Merck, Darmstadt, Germany). Thin-layer chromatography was carried out with pre-coated MERCK silica gel 60 PF254 (Merck, Darmstadt, Germany); the spots were visualised under UV light (254 and 365 nm) and further by spraying with anisaldehyde and then heating until charred.

3.2. Plant material

The roots of *M. minutum* were collected in Amphoe Muang, Khon Kaen Province, Thailand, in June 2011. The plant material was identified by Prof. Pranom Chantaranothai, Department of Biology, Khon Kaen University, Thailand, where a voucher specimen (S. Kanokmedhakul 10) was deposited.

3.3. Extraction and isolation

Air-dried roots of *M. minutum* (1.1 kg) were grounded into powder and then extracted successively with hexane, EtOAc and MeOH. Removal of solvents from each extract under reduced pressure gave crude hexane (14.5 g, 1.32%), EtOAc (123.2 g, 11.20%) and MeOH (66.2 g, 6.02%) extracts. The EtOAc extract (100.0 g) was separated over silica gel CC, eluted with gradient systems of hexane-EtOAc and EtOAc-MeOH to give ten fractions, EF_1–EF_{10}. Fraction EF_2 was purified by silica gel CC, gradually eluted with CH_2Cl_2–EtOAc and further purified by preparative TLC using CH_2Cl_2-EtOAc (99 : 1) as developing solvent to give **2** as a colourless oil (65.0 mg, $R_f = 0.77$). Fraction EF_7 was subjected to silica gel CC and gradually eluted with a gradient of hexane-EtOAc to give five subfractions, $EF_{7.1}$–$EF_{7.5}$. Subfraction $EF_{7.2}$ was purified by silica gel CC, eluted with a gradient system of CH_2Cl_2–EtOAc to afford **3** as a white solid (501.2 mg). The solid of fraction $EF_{7.4}$ was recrystallised with hexane-EtOAc (30:70) to yield **4** as a white solid (175.4 mg). Fraction EF_8 was dissolved in EtOAc, and the precipitate was filtered to obtain **5** as a white solid (2.7 g). The filtrate was evaporated *in vacuo* and a residue (10.5 g) was separated over silica gel flash column chromatography, eluted with gradient systems of hexane-EtOAc and EtOAc-MeOH to give nine fractions, $EF_{8.1}$–$EF_{8.9}$. Fraction $EF_{8.4}$ was dissolved in EtOAc and the precipitate was filtered to yield **6** as a white solid (1.9 g). Fraction $EF_{8.5}$ was purified by preparative TLC using hexane-EtOAc (60:40) as developing solvent to yield **1** as a colourless oil (25.7 mg, $R_f = 0.37$). Fraction EF_9 was separated on silica gel CC, eluted with a gradient system of hexane-EtOAc to give nine subfractions, $EF_{9.1}$–$EF_{9.9}$. Subfraction $EF_{9.2}$ was purified by preparative TLC using CH_2Cl_2-EtOAc (40:60) as developing solvent to yield **7** as a colourless oil (25.7 mg, $R_f = 0.44$). Subfraction $EF_{9.4}$ was subjected to silica gel CC and eluted with a gradient system of hexane-EtOAc to give four subfractions, $EF_{9.4.1}$–$EF_{9.4.4}$. Subfraction $EF_{9.4.2}$ was further purified by preparative TLC using hexane-EtOAc (40 : 60) as developing solvent to yield **8** as a white solid (591.7 mg, $R_f = 0.24$). The solid in subfraction $EF_{9.4.3}$ was recrystallised from MeOH to yield **9** as a white solid

(95.2 mg). The MeOH extract (60.2 g) was separated on silica gel CC, eluted with gradient systems of hexane-EtOAc and EtOAc-MeOH to give six fractions, MF_1–MF_6. Fraction MF_4 was separated by CC, eluted with a gradient system of hexane-EtOAc to give ten subfractions, $MF_{4.1}$–$MF_{4.10}$. Subfraction $MF_{4.5}$ was purified by silica gel CC, eluted with a gradient system of hexane-CH_2Cl_2 and further purified by preparative TLC using CH_2Cl_2-EtOAc (95 : 5) as developing solvent to yield **12** as a white solid (8.8 mg, $R_f = 0.34$). Fraction $MF_{4.8}$ was separated by silica gel CC, eluted with a gradient system of CH_2Cl_2–EtOAc to give **11** as a white solid (4.6 mg). Fraction MF_5 was dissolved in EtOAc and the precipitate was filtered to yield an additional amount of **5** (49.3 mg). Fraction MF_6 was purified by silica gel CC, eluted with a gradient system of hexane-EtOAc and preparative TLC using hexane-EtOAc (95:5) as developing solvent to yield **10** as a white solid (9.0 mg, $R_f = 0.33$).

Minutuminolate (**1**): colourless oil: $[\alpha]^{26}_D$ + 52.0 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε): 324 (4.17), 260 (3.68) nm; IR (KBr) ν_{max}: 2911, 2843 (C–H, s), 1727 (C=O, s), 1601 (C=C, w), 1495, 1366 (C–H, w), 1083 (C–O, w) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Table S1; HRESITOFMS *m/z* 381.1318 $[M + Na]^+$ (Calcd *m/z* 381.1314).

3.4 Cytotoxicity assay

Cytotoxic assays against human epidermoid carcinoma (KB), human small cell lung cancer (NCI-H187), and human breast cancer (MFC-7) cell lines were performed employing the colorimetric method as described by Skehan and co-workers (Skehan et al. 1990). The reference substances were ellipticine and doxorubicin.

4. Conclusion

Chemical investigation of the EtOAc and MeOH extracts from the roots of *M. minutum* led to the isolation of twelve compounds with classical coumarin characteristic of the genus. In this report, one new coumarin, minutuminolate (**1**), and eleven known compounds (**2**–**12**) were isolated. This is the first report of coumarins **6** and **7** isolated from *Micromelum* species. It was found that compounds **2**, **5**, **8**, **9** and **10** have previously been isolated from the fruits of *M. minutum*. In addition, compound **5** is the main chemical constituent isolated from both fruits and roots of this plant.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Short communication

Cytotoxic and antimalarial constituents from aerial parts of *Sphaeranthus indicus*

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ABSTRACT

Two new eudesmanolide type sesquiterpenes, indicusalactone (**1**) and (−)-oxyfrullanolide (**2**), along with twelve known compounds (**3**–**14**), were isolated from the aerial parts of *Sphaeranthus indicus*. The structures of these compounds were established on the basis of their 1D and 2D NMR spectroscopic data. Compounds **1–4** and **12–14** showed antimalarial activity against *Plasmodium falciparum* with IC_{50} values ranging from 2.32 to 6.47 μg/mL. In addition, compounds **2**–**5** showed cytotoxicity against cancer cell lines, KB, NCI-H187 and MCF-7 with IC_{50} values within the range 1.23–46.19 μg/mL.

1. Introduction

Sphaeranthus indicus belongs to the aroma family Asteraceae (Compositae), is 20–50 cm in height, and grows widely in the rice-fields of the northern and northeastern parts of Thailand. It is called 'Matomsuea' in Thai and is used traditionally as a decoction for the treatment of skin diseases and nervous depression (Galani et al., 2010; Smitinand, 2014). Phytochemical investigations on *Sphaeranthus* species demonstrated the presence of bioactive eudesmanolides sesquiterpene lactones (Atta et al., 1989; Ruangrungsi et al., 1989; Shekhani et al., 1991; Rojatkar and Nagasampagi, 1992; Rojatkar et al., 1994; Sohoni et al., 1998; Pujar et al., 2000; Jadhav et al., 2007; Mishra et al., 2016), glycosides, flavonoids (Mishra et al., 2007; Yadava and Kumar, 1999), and essential oil (Kaul et al., 2005). As part of our search for bioactive compounds from Thai medicinal plants, we noted that the hexane and EtOAc extracts from the aerial parts of *S. indicus* showed cytotoxicity against cancer cells, NCI-H187 (IC_{50} 6.72 and 11.64 μg/mL, respectively) and MCF-7 (IC_{50} 8.57 and 13.49 μg/mL, respectively). However, all crude extracts showed no activity against *Plasmodium falciparum* and *Mycobacterium tuberculosis* (both $IC_{50} \geq 90$ μg/mL). We report herein the isolation, characterization and biological activities of two new compounds (**1**–**2**) (Fig. 1) along with twelve known compounds (−)-frullanolide (**3**) (Ruangrungsi et al., 1989), 7-hydroxyfrullanolide (**4**) (Atta et al., 1989), 3α-hydroxy-eudesm-4-en-12,6β-olide (**5**) (Oksuz and Topcu, 1992), ilicic acid (**6**) (Guilhon and Müller, 1998), 5α-hydroxy-4α,15-dihydrocostic acid (**7**) (Hegazy et al., 2014), eudesmanolide dimer (**8**) (Gokaraju et al., 2013), 2,5-dimethoxy-*p*-cymene (**9**) (Kaul et al., 2005), pangelin (**10**) (Thanh et al., 2004), luteolin 4′-methyl ether (**11**) (Zhi et al., 2012), squalene (**12**) (Huang et al., 2009), 3,5-di-*O*-caffeoylquinic acid methyl ester (**13**) and 3,4-di-*O*-caffeoylquinic acid methyl ester (**14**) (Hu et al., 2014) (Fig. S19 in Supplementary data) It should be noted that this is the first report of compound **12** from *S. indicus*, while compounds **5**, **7**, **10**, **11**, **13** and **14** are reported for the first time from the *Sphaeranthus* species.

2. Results and discussion

Compound **1** was obtained as a white solid, with the molecular formula $C_{15}H_{18}O_4$ assigned from the HRESITOFMS (*m*/*z* 285.1091 $[M+Na]^+$), indicating seven degrees of unsaturation. The IR spectrum indicated the presence of lactone groups (1774 and 1761 cm^{-1}). The ^1H NMR spectral data (Table 1) indicated two singlet resonances of methyl protons at δ_H 1.09 (H_3-14) and 1.24 (H_3-15), alkene methylene protons at δ_H 6.05 and 6.34 (both brs, H_2-12), together with five methylene protons at δ_H 1.70 and 1.46 (both m, H_2-1), 2.08 and 1.66 (both m, H_2-2), 1.63 and 1.58 (both m, H_2-5), 2.50 and 1.90 (both m, H_2-7) as well as δ_H 1.85 and 1.60 (both

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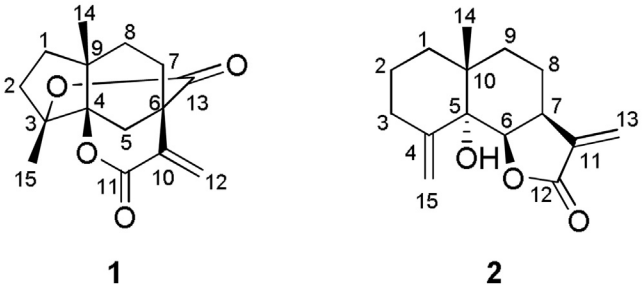

Fig. 1. Structures of compounds 1 and 2.

Table 1
^{1}H and ^{13}C NMR spectral data for compounds **1** and **2** ($CDCl_3$).[a]

Position	1		2	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	1.70 (m), 1.46 (m)	32.3	1.88 (dt, 13.6, 4.4)	31.7
2	2.08 (m), 1.66 (m)	32.9	1.57 (m)	21.8
3		87.0	2.52 (dt, 13.6, 6.0), 2.19 (dd, 13.6, 4.4)	32.1
4		92.9		147.0
5	1.63 (m)[b], 1.58 (m)[b]	14.9		74.9
6		61.7	4.26 (d, 4.8)	80.0
7	2.50 (m), 1.90 (m)	35.9	3.25 (ddd, 12.0, 6.8, 4.8)	39.1
8	1.85(m), 1.60 (m)	38.9	1.71 (m), 1.57 (m)	24.3
9		45.2	1.09 (m)	36.5
10		136.8		36.9
11		168.3		141.7
12	6.34 (brs), 6.05 (brs)	124.9		170.7
13		174.5	6.07 (brs), 5.55 (brs)	119.4
14	1.09 (s)	23.1	0.98 (s)	18.8
15	1.24 (s)	26.3	5.28 (brs), 5.01 (brs)	111.7

[a] Values in parentheses are coupling constants in Hz.
[b] Overlapping signals.

m, H_2-8). In turn, the ^{13}C NMR data and HSQC experiment showed 15 carbon signals including those for two oxygenated sp^3 carbons (δ_C 87.0 and 92.9), olefinic carbons (δ_C 124.9 and 136.8), two carbonyl lactone groups (δ_C 168.3 and 174.5), two methyl groups (δ_C 23.1 and 26.3), five methylene carbons and three quaternary carbons (Table 1). Analysis of the COSY and HMQC spectra revealed the partial connections between H-1↔H-2 and H-7↔H-8 which were connected further based on long-range HMBC correlations (Fig. 2a). The HMBC spectrum of **1** showed correlations of the olefinic methylene protons at δ_H 6.34 and 6.05 (H_2-12) to the lactone carbonyls at δ_C 168.3 (C-11) and 174.5 (C-13) and two quaternary carbons at δ_C 61.7 (C-6) and 136.8 (C-10). The methylene proton H_2-5 showed correlation to the quaternary oxygenated carbon at δ 92.9 (C-4), while the methylene protons H_2-7 showed correlations to the lactone carbonyl carbon at δ_C 174.5 (C-13) and a quaternary carbon at δ_C 61.7 (C-6). Furthermore, significant correlations of the methyl groups at δ_H 1.24 (H_3-15) to C-2, C-3 and C-4 proved the location of one lactone ring at C-3 and C-4, respectively. The relative stereochemistry of **4** was determined by analysis of the coupling constants and by NOESY correlations of those protons (Fig. 2b). On the basis of the above data, the structure of **1** was assigned as a new rearranged eudesmanolide derivative, and it has been named indicusalactone.

Compound **2** was obtained as a white solid and its molecular formula $C_{15}H_{20}O_3$ deduced from the HRESITOFMS (m/z 271.1302 $[M+Na]^+$), indicating six degrees of unsaturation. The IR spectrum indicated the presence of a hydroxyl group (3447 cm^{-1}) and a γ-lactone ring (1764 cm^{-1}). Its ^{1}H NMR spectrum (Table 1) showed a methyl singlet at δ_H 0.98 along with two sets of protons at δ_H 6.07 and 5.55 (each 1H, brs, H_2-13), δ_H 5.28 and 5.01 (each 1H, brs, H_2-15), indicating that the compound is a sesquiterpene with two exomethylene groups, two methine protons δ_H 4.26 (d, J = 4.8 Hz, H-6) and 3.25 (ddd, J = 12.0, 6.8, 4.8 Hz, H-7) as a *cis*-fused lactone ring (Jakupovic et al., 1990). The ^{13}C NMR and DEPT spectra indicated 15 carbons attributable to one methyl, seven methylenes (including two olefinic methylene protons), two methines, and five quaternary (including one carbonyl) carbons. The ^{1}H NMR spectral data (Table 1) of **2** was identical to the data of the known compound (+)-oxyfrullanolide isolated from the *Frullania* species of liverwort (Asakawa et al., 1976). 1D and 2D NMR spectroscopic analyses (Fig. 3a) indicated that both compounds have the same planar structure and the same relative configuration. The relative configuration at C-6 and C-7 were determined from the coupling constant of H-6 (4.4 Hz) and the correlations between H-6 and H-7 in the NOESY spectrum (Fig. 3b). However, the optical rotation of **2** ($[\alpha]^{26}_D$ −68.0) was opposite in sign to (+)-oxyfrullanolide ($[\alpha]^{25}_D$ +71.0) (Asakawa et al., 1976), suggesting that these two compounds are enantiomers. On the basis of the above data the structure of **2** was assigned as a new eudesmanolide, (−)-oxyfrullanolide. Interestingly, all structurally similar compounds to oxyfrullanolide from *S. indicus* also possess a negative optical rotation. It is noteworthy that the (+)-enantiomer was isolated from liverwort, a bryophyte, whilst our bush tree led to the (−)-enantiomer. This difference might arise from the evolutionary distance separating those two families.

Compounds **1–14** were tested for bioactivity, and results are given in Table 2. Compounds **1–4** and **12–14** showed antimalarial activity against *P. falciparum* with IC_{50} values in the range of 2.32–6.47 μg/mL. Compounds **1** and **3** exhibited strong cytotoxicity against KB cell lines with IC_{50} values of 2.74 and 3.79 μg/mL, respectively. Besides, compounds **1**, **3** and **4** showed cytotoxicity against NCI-H187 cell lines with IC_{50} values of 4.81, 5.63 and 1.23 μg/mL, respectively. Interestingly, compounds **1** and **4**

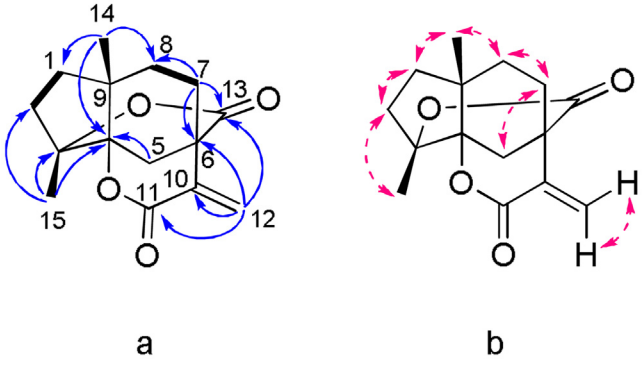

Fig. 2. a) COSY (bold line) and selected HMBC (H→C) correlations of **1**. b) Key NOESY (·····) correlations of **1**.

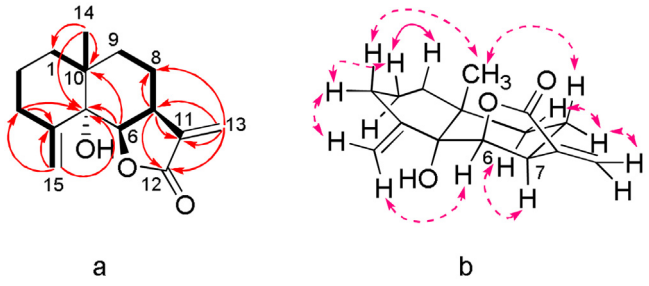

Fig. 3. a) COSY (bold line) and selected HMBC (H→C) correlations of **2**. b) Key NOESY (·····) correlations of **2**.

Table 2
Biological activities of the isolated compounds.

compound	antimalarial (IC_{50}, μg/mL)	antimycobacterial (MIC, μg/mL)	cytotoxicity (IC_{50}, μg/mL)		
			KB[a]	NCI-H187[b]	MCF7[c]
1	2.87	inactive	2.74	4.81	5.74
2	3.82	inactive	12.80	14.38	44.41
3	6.47	inactive	3.79	5.63	42.12
4	2.49	12.50	20.71	1.23	2.92
5	inactive	nd[d]	43.30	nd[d]	23.12
6	inactive	inactive	inactive	inactive	inactive
7	inactive	nd[d]	inactive	nd[d]	inactive
8	inactive	nd[d]	inactive	nd[d]	inactive
9	inactive	inactive	inactive	inactive	inactive
10	inactive	nd[d]	inactive	nd[d]	inactive
11	inactive	nd[d]	inactive	nd[d]	inactive
12	2.32	inactive	inactive	15.50	46.19
13	2.39	nd[d]	inactive	nd[d]	inactive
14	2.90	nd[d]	inactive	nd[d]	inactive
dihydroarte-misinin	0.002				
mefloquine	0.0247				
isoniazid		0.047			
ellipticine			1.21	0.935	
doxorubicin			0.855	0.057	7.90

[a] Humanepidermoid carcinoma in the mouth.
[b] Human lung cancer cell.
[c] Human breast cancer cell.
[d] Not determined.

exhibited strong activity against MCF-7 cell lines with IC_{50} values of 5.74 and 2.92, lower than the control drug doxorubicin (7.90 μg/mL). Among these, only **4** showed antimicrobacterial activities against *M. tuberculosis*, with MIC value of 12.50 μg/mL, which corresponds to several previous reports of its antimicrobial activities (Ata et al., 2009). The sesquiterpene lactone 4 has also previously been reported to show anti-inflammatory (Fonseca et al., 2010) and cytotoxic activities (Nahata et al., 2013).

3. Experimental

3.1. General experimental procedures

Melting points were determined using an Electrothermal IA9200 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK). Optical rotations were measured on a JASCO-DIP-1000 digital polarimeter (JASCO Inc., USA) and UV spectra were recorded using an Agilent 8453 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). IR spectra were obtained using a Bruker Tenser 27 spectrophotometer (Bruker, Germany). NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer (Varian Inc., USA) using $CDCl_3$, CD_3OD and DMSO-d_6 as solvents. The internal standards were referenced from the residue of those solvents. The HR-ESI-TOF-MS were recorded on a Bruker micrOTOF mass spectrometer (Bruker, Germany). Column chromatography was carried out on MERCK silica gel 60 (230–400 mesh) (Merck, Darmstadt, Germany). Thin-layer chromatography was carried out with pre-coated MERCK silica gel 60 PF254 (Merck, Darmstadt, Germany); the spots were visualized under UV light (254 and 365 nm) and further stained by spraying with anisaldehyde and then heated until charred.

3.2. Plant material

The aerial parts of *S. indicus* were collected in Amphoe Kasetsomboon, Chaiyaphum Province, Thailand, in April 2013. The plant material was identified by Prof. Pranom Chantaranothai, Department of Biology, Khon Kaen University, Thailand where a voucher specimen (R. Lekphrom kku006) was deposited.

3.3. Extraction and isolation

Air-dried aerial parts of *S. indicus* (2.0 kg) were ground to powder and then extracted successively at room temperature with hexane, EtOAc and MeOH three times each (4L × 3), respectively. Removal of solvents under reduced pressure gave crude hexane (102 g, 2.6%), crude EtOAc (150 g, 7.5%) and crude MeOH (300 g, 15.0%) extracts. The crude hexane extract (102 g) was separated over silica gel CC, eluted with a gradient systems of EtOAc-hexane and MeOH-EtOAc to give seven fractions, HF_1-HF_7. Fraction HF_3 was recrystallized from hexane to yield a white solid of **3** (705.0 mg). The crude EtOAc extract (150 g) was subjected initially to silica gel CC, eluted with the same gradient system as the hexane extract above to give seventeen fractions, EF_1-EF_{17}. Fraction EF_1 was purified by preparative TLC using EtOAc-hexane (1:9) as developing solvent to yield a light yellow oil of **12** (10.5 mg). Fraction EF_2 was separated on silica gel FCC, eluting with hexane to give six subfractions, $EF_{2.1}$-$EF_{2.6}$. Subfraction $EF_{2.3}$ was purified on silica gel FCC, eluted with EtOAc-hexane to yield a white solid of **11** (61.2 mg). Fraction EF_4 was separated on silica gel FCC, eluted with an isocratic system of CH_2Cl_2-hexane (1:9) to give fourteen subfractions, $EF_{4.1}$-$EF_{4.14}$. Subfraction $EF_{4.10}$ was recrystallized from hexane to yield a white solid of **2** (5.2 mg). Fraction EF_5 was separated by FCC, eluted with a gradient system of EtOAc-hexane to give six subfractions, $EF_{5.1}$-$EF_{5.6}$. Subfraction $EF_{5.4}$ was recrystallized from hexane to give an additional amount of **2** (30.0 mg). Subfraction $EF_{5.5}$ was further separated by FCC, eluted with an isocratic system of EtOAc-hexane (1:5) to give a white powder of **5** (7.8 mg). Subfraction $EF_{5.6}$ was separated by FCC, eluted with an isocratic system of acetone-hexane (1:9) to give four subfractions, $E_{5.6.1}$-$E_{5.6.4}$. Subfractions $EF_{5.6.3}$ and $EF_{5.6.4}$ were recrystallized from hexane to give a white solid of **1** (35.0 mg) and a white solid of **8** (31.0 mg), respectively. Fraction EF_7 was chromatographed on silica gel FCC, eluted with EtOAc-CH_2Cl_2-hexane (1:4:5) to give three subfractions, $EF_{7.1}$-$EF_{7.3}$. Subfraction $EF_{7.1}$ was purified by preparative TLC using CH_2Cl_2-hexane (2:3) as eluent to yield a brown viscous oil of **4** (12.6 mg). Subfraction $EF_{7.2}$ was separated by FCC, eluted with an isocratic system of EtOAc-hexane (3:7) to give seven subfractions, $EF_{7.2.1}$-$EF_{7.2.7}$. Subfraction $EF_{7.2.1}$ led to the isolation of an additional amount of **4** (2.8 g). Subfraction $EF_{7.2.2}$ was recrystallized from CH_2Cl_2 to give a yellow solid of **9** (10 mg). Subfraction $EF_{7.3}$ was separated by silica gel FCC, eluted with an isocratic system of EtOAc-hexane (1:1) to give six subfractions, $EF_{7.3.1}$-$EF_{7.3.6}$. Subfraction $EF_{7.3.6}$ was separated by silica gel FCC, eluted with an isocratic system of EtOAc-hexane (1:1) to yield a brown solid of **7** (6.5 mg). Fraction EF_8 was subjected to silica gel FCC, eluted with an isocratic system of EtOAc-CH_2Cl_2 (1:9) to yield a white solid of **6** (4.0 mg). Fraction EF_{14} was separated by FCC, eluted with a gradient system of MeOH-CH_2Cl_2 to give a yellow solid of **10** (8.0 mg). The MeOH extract (300 g) was separated on silica gel CC, eluted with a gradient system of hexane-EtOAc and EtOAc-MeOH to give six fractions, MF_1-MF_6. Fraction MF_4 was separated by FCC, eluted with an isocratic system of EtOAc-hexane (4:1) to give six subfractions, $MF_{4.1}$-$MF_{4.6}$. Subfraction $MF_{4.4}$ was further subjected to chromatography using a silica gel FCC, eluted with an isocratic system of EtOAc-hexane (7:3) to give seven subfractions, $MF_{4.4.1}$-$MF_{4.4.7}$. Subfractions $MF_{4.4.2}$ and $MF_{4.4.4}$ led to the isolation of a yellow gum of **13** (100.0 mg) and a yellow gum of **14** (20.5 mg), respectively.

3.3.1. Indicusalactone (**1**)

mp 106–107 °C; $[\alpha]^{26}_D$ −7.6 (*c* 1.0, $CHCl_3$); IR (neat); ν_{max} 2938, 1774, 1761, 1289, 1219, 1137 and 1052 cm^{-1}; ESITOFMS *m/z* 285.1091 $[M+Na]^+$ (calcd for $C_{15}H_{18}O_4$ + Na: 285.1097).

*3.3.2. (−)-Oxyfrullanolide (**2**)*

mp 173–174 °C; $[\alpha]^{26}_{D}$ −68.0 (*c* 1.0, $CHCl_3$); IR (neat): ν_{max} 3447, 2929, 2868, 1764, 1457, 1380 and 1144 cm^{-1}; ESITOFMS *m/z* 271.1302 $[M+Na]^+$ (calcd for $C_{15}H_{20}O_3$ + Na: 271.1305).

3.4. Bioassays

3.4.1. Antimalarial assay

Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug resistant strain), using the method of Trager and Jensen (1976). Quantitative assessment of malarial activity *in vitro* was determined by means of the microculture radio isotope technique based up on the method described by Desjardins (Desjardins et al., 1979). The inhibitory concentration (IC_{50}) represents the concentration which causes 50% reduction in parasite growth as indicated by the *in vitro* incorporation of [^{3}H]-hypoxanthine by *P. falciparum*. The standard compounds were dihydroartemisinin and mefloquine (Table 2).

3.4.2. Antimycobacterial assay

The antimycobacterial activity was assessed against *Mycrobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). The standard drug isoniazid was used as the reference compound (Table 2).

3.4.3. Cytotoxicity assay

The cytotoxic assays against human epidermoid carcinoma (KB), human small cell lung cancer (NCI-H187) and human breast cancer (MCF-7) cell lines were performed employing the colorimetric method as described by Skehan (Skehan et al., 1990). The reference substances were ellipticine and doxorubicin (Table 2).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.08.001.

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